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A Micro Method for Separating Free and Esterified Vitamin A

By E. EDEN, *Dunn Nutritional Laboratory and Institute of Animal Pathology, University of Cambridge*

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Glover, Goodwin & Morton (1947) have described a chromatographic method for separating free and esterified vitamin A, using bone meal as adsorbent. The method was developed for the separation of 600–1800 i.u. of vitamin A per column. For the estimation of free and esterified vitamin A in blood and lymph it is, however, often necessary to be able to separate smaller amounts, i.e. a few international units. Several other methods of separation have been published. In the earliest of these, phase separation was used (for a review of the literature see Glover *et al.* 1947), but recently chromatography has been employed by most workers (Table 1). Molecular distillation (Gray, Hickmann & Brown, 1940) and fluorescence methods (Sobotka, Kann & Winternitz, 1944; Kascher & Baxter, 1945) have also been applied.

Table 1. *Adsorbents and eluting agents used for the separation of free and esterified vitamin A*

Adsorbent	Solvents and eluting agent for: Vitamin A ester	Vitamin A alcohol	Reference
Al_2O_3	Hexane	Ethanol	Hoch & Hoch (1946)
Al_2O_3	Acetone (2%) in hexane	Ethanol (8%) in hexane	Ganguly, Kon & Thompson (1947)
Al_2O_3	Acetone (4%) in light petroleum	Estimated by deduction from total	Parrish, Wise & Hughes (1947)
Al_2O_3	Ethylene dichloride	Estimated by deduction from total	Reed, Wise & Frunat (1944)
Al_2O_3	Light petroleum	Acetone (20%) in light petroleum	Present investigation
$Al_2(SO_4)_3$ (on filter paper)	Light petroleum	Light petroleum	Datta & Overell (1949)
Bone meal	$CHCl_3$ (2·5%) in light petroleum	Acetone	Glover *et al.* (1947)
Kieselguhr (Hyflo Super Cel)	Light petroleum	Light petroleum	Wilkes (1946)
Silicic acid	Light petroleum	$CHCl_3$	Swain (1943)
MgO + celite	Light petroleum	$CHCl_3$ (5%) in light petroleum	Narod & Verhagen (1948)

Of these various methods, chromatography is the most readily adapted to small-scale work. On a column of aluminium oxide, vitamin A alcohol travels with the hydroxy carotenoids, whereas esterified vitamin A is less strongly adsorbed, and moves with the hydrocarbon carotenoids. Vitamin A alcohol and its esters are almost colourless, but the accompanying carotenoids, when present, serve as a guide to separation. Hoch & Hoch (1946), working on human sera, were able to take advantage of this, but, in the present work, the extracts did not contain these carotenoids and the main problem was to find suitable 'indicators' or an alternative mode of recognition. Lycopene and Sudan III were tried but gave colours with antimony trichloride. Ultraviolet illumination also failed, as the amount of vitamin A present (a few micrograms) was not always sufficient to show a clear fluorescent zone on the column. Eventually, by using constant amounts of adsorbents and eluents it was possible to do without indicators. The efficiency of separation was checked in each case by collecting a middle fraction between the vitamin A alcohol and the ester fractions; the separation was only considered satisfactory when this fraction did not contain any vitamin A.

EXPERIMENTAL

Principle of method. A small column of Al_2O_3 was used in which vitamin A alcohol was adsorbed from a light petroleum solution, whereas the ester passed straight through and was collected as 'Fraction I'. To check whether all esters had been removed, a second fraction ('Fraction II') was collected by washing the column with light petroleum. Then the vitamin A alcohol was eluted with a solution of 20% acetone in light petroleum and collected as 'Fraction III'.

Preparation of the column. Glass tubes of internal diameter 6 mm. and height 100–130 mm. were plugged with cotton wool above a 1–2 mm. diameter constriction. Al_2O_3 (British Drug Houses Ltd.) and light petroleum were mixed

and poured into the tube to give on settling a 15 mm. layer of solid; the surface of the column was always covered with liquid, and the rate of flow without suction was 20–60 drops/min.

Chromatography. The extract containing the vitamin A (4–40 i.u.) was evaporated to dryness under reduced pressure. (For details of extracting vitamin A from biological tissues see Eden & Sellers, 1950.) The residue was made up in 1 ml. of light petroleum and the solution was transferred to the column. The flask was rinsed three times with 1, 2 and 2 ml. of light petroleum respectively. The filtrate from the original 1 ml. and the three washings were collected as 'Fraction I' containing the vitamin A esters. The column was then washed with an additional 2 ml. of light petroleum. If this middle fraction, containing the 2 ml. of washings, was found to contain no vitamin A, the separation was considered satisfactory. The third fraction containing the vitamin A alcohol was obtained by eluting the column twice with 2 ml. of 20% acetone in light petroleum. Each fraction was evaporated under reduced pressure and then dissolved in $CHCl_3$.

Estimation of vitamin A. The intensity of the blue colour in the $SbCl_3$ reaction was measured in a single photocell absorptiometer (Eden & Sellers, 1949).

RESULTS

Efficiency of separation of artificial mixtures

Table 2 shows the separation obtained. The free vitamin A used was a crystalline product obtained from Roche Products Ltd. and the vitamin A ester a concentrate prepared by Distillation Products Inc. Solutions of these compounds in light petroleum were mixed and subjected to chromatographic separation. The recovery of both vitamin A ester and alcohol was usually within 5% of the expected value and the 'middle' fraction contained no more than traces of vitamin A.

Recovery and separation of vitamin A alcohol and ester added to plasma extracts

Extracts of plasma, intestinal contents and mucosa in light petroleum were chromatographed after addition of small quantities of vitamin A alcohol or ester. Control extracts without the addition of vitamin A were put through the same procedure. Table 3 shows that the separation is also satisfactory in the presence of biological extracts.

SUMMARY

1. A micro method using aluminium oxide as adsorbent has been devised for separating vitamin A alcohol from the esterified form. Under the conditions of the method, vitamin A ester in light petroleum passed through the column, whereas vitamin A alcohol was adsorbed and could be eluted with 20% acetone in light petroleum.

Table 2. *Chromatographic separation of vitamin A alcohol and esters*

Vitamin A ester			Vitamin A alcohol			Percentage of total vitamin A in the 'middle' fraction
Added (i.u.)	Recovered (i.u.)	Recovery (%)	Added (i.u.)	Recovered (i.u.)	Recovery (%)	
15·0	14·4	96	Nil	Nil	—	0·0
11·0	11·2	102	Nil	Nil	—	1·0
10·1	9·4	93	Nil	Nil	—	0·7
5·1	5·2	102	3·15	3·1	98	0·0
5·1	5·13	101	6·3	6·6	104	0·0
2·52	2·56	102	6·3	5·8	92	0·1
10·97	10·75	98	20·15	19·1	95	0·4
Nil	Nil	—	20·15	19·8	98	0·1
Nil	Nil	—	9·8	10·2	104	0·0

Table 3. *Recovery of vitamin A alcohol and ester added to biological extracts*

	Vitamin A ester				Vitamin A alcohol			
Nature of extract	Original (i.u.)	Added (i.u.)	Found in ester fraction (i.u.)	Recovery of added vitamin A (%)	Original (i.u.)	Added (i.u.)	Found in alcohol fraction (i.u.)	Recovery of added vitamin A (%)
Plasma	0·11	11·5	11·59	101	1·42	0	1·40	—
,,	1·51	10·1	9·24	91	6·79	0	6·99	—
,,	0·07	7·6	7·12	94	12·7	0	12·3	—
,,	0·07	9·1	8·9	98	12·3	0	11·7	—
,,	0·33	0	0·41	—	5·20	12·7	17·0	93
,,	3·18	0	3·49	—	7·19	25·4	35·9	113
Intestinal contents	1·87	9·3	9·3	100	1·68	23·4	22·6	97
Intestinal mucosa	0	22·7	22·5	99	0	13·5	13·2	99

2. The separation proved to be satisfactory either for artificial mixtures or biological extracts fortified with vitamin A alcohol or ester.

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Hydrolysis and Esterification of Vitamin A during Absorption

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The absorption of vitamin A by the lymph stream has been demonstrated in several species, including man (Drummond, Bell & Palmer, 1935), dogs (McCoord, Breese & Baum, 1943), rats (Popper & Volk, 1944; Radice & Herraiz, 1947), sheep (Eden & Sellers, 1949), and bovines (Eden & Sellers, 1949; Goodwin & Gregory, 1948). Further studies on the mechanism of vitamin A absorption have been made by Gray, Morgareidge & Cawley (1940) who have shown that vitamin A esters are hydrolysed in the intestinal tract of the rat. In the present work the fate of free and esterified vitamin A during absorption by bovines and sheep has been studied.

EXPERIMENTAL

The procedure adopted was to determine the relative concentration of vitamin A alcohol and ester in the intestinal content, mucosa, lymph and blood after dosing with one or other form of the vitamin. The interval between dosing and slaughter was chosen as 4 hr., as previous experiments (Eden & Sellers, 1949) had shown that, although the rate of absorption of vitamin A was variable, it most commonly reached its maximum then.

The experimental animals were ten adult sheep and eleven calves. All the sheep and seven of the calves were about a year old; four newborn calves were also studied, to see if absorption was the same in them as in older animals.

Preparation of dose. Vitamin A ester concentrates (from fish-liver oils) in which over 95 % of the vitamin was esterified, were obtained from Distillation Products Inc. Vitamin A alcohol was prepared by saponification of halibut-liver oil, and was compared chromatographically with a crystalline preparation of vitamin A alcohol obtained from Roche Products Ltd. Over 95 % of the vitamin A in the liver oil unsaponifiable fraction was free alcohol. The materials were made up for dosing by emulsifying the vitamin A with reconstituted separated milk and arachis oil in a Waring blender. The animals received about 5000 i.u. of vitamin A/kg. of body weight, and the total volume of the dose was 200–400 ml. according to the size of the animal.

Collection of materials for estimation. Before dosing and at slaughter, samples of jugular blood were collected. At slaughter the intestinal lymph glands were removed, sliced and centrifuged, the supernatant liquid drawn off and its vitamin A content determined. The intestinal contents were obtained from the duodenum and the jejunum. These portions of intestine were cut open and washed repeatedly with 0·9 % saline in order to remove any adhering contents. The samples of mucosa were removed with a scalpel from the connective and muscular tissue.

Chemical methods

Methods of extraction. Plasma (6 ml.), or lymph (1 ml.) were treated according to the method of Yudkin (1941), which consists in precipitating the proteins with ethanol and extracting the vitamin with light petroleum (b.p. 40–60°). This method was slightly modified for the extraction of intestinal contents and mucosa. The intestinal contents (3 ml.) were diluted with an equal volume of distilled water, 3 ml. of ethanol were added, then the mixture was extracted with 12 ml. of light petroleum. After shaking and centrifuging, 10 ml. of light petroleum were withdrawn and the sample re-extracted with another 10 ml. of light petroleum. Samples of intestinal mucosa (4 g.) were weighed into 50 ml. thick-walled glass beakers, cut up finely, and then ground with a little quartz in presence of 4 ml. of ethanol. The contents of the beaker were transferred to a 25 ml. centrifuge tube and the beaker was rinsed twice with 2 ml. of distilled water. To the centrifuge tube 12 ml. of light petroleum were added and the rest of the procedure was the same as that used for plasma.

Separation of vitamin A alcohol and ester. A chromatographic separation was effected by a modified method of Hoch & Hoch (1946). A small column of Al_2O_3 was used, and the conditions were so adjusted that vitamin A alcohol was adsorbed from a light petroleum solution while the esters passed through unabsorbed. Vitamin A alcohol was then eluted with 20% acetone in light petroleum. For further details of this method see Eden (1950).

Estimation of vitamin A. The intensity of the blue colour in the $SbCl_3$ reaction for the estimation of vitamin A was measured in a single photocell absorptiometer (Eden & Sellers, 1949).

Table 1. *Concentrations of Vitamin A alcohol and ester in the intestinal contents of calves*

Calf no.	Duodenum (D) or small intestine (S)	Vitamin A (i.u./ml.) Alcohol fraction	Ester fraction	Total	Percentage of total vitamin A esterified
	Vitamin A ester (5000 i.u./kg. of body wt.) given 4 hr. before slaughter				
55	D and S	3·3	21·0	24·3	86
56	D and S	5·0	2·39	7·39	32
57	D	0·84	0·94	1·78	53
43*	D	3·9	0·07	3·97	3
43	S	3·1	0·16	3·17	5
46*	D	28·8	21·9	50·70	42
46	S	10·3	64·2	74·50	86
47*	D	5·85	40·0	45·85	88
47	S	14·85	39·3	54·15	72
48*	D	6·08	15·1	21·18	71
48	S	0·67	0·8	1·45	55
				Average	54
	Vitamin A alcohol (5000 i.u./kg. of body wt.) given 4 hr. before slaughter				
49	D	3·6	0·25	3·85	6
50	D and S	32·2	2·2	34·4	6
	Untreated animals				
52	D and S		0·03		
53	D and S		0·03		

* Newborn calves.

Table 2. *Concentrations of vitamin A alcohol and ester in the intestinal contents of sheep*

Sheep no.	Duodenum (D) or small intestine (S)	Vitamin A (i.u./ml.) Alcohol fraction	Ester fraction	Total	Percentage of total vitamin A esterified
	Vitamin A ester (5000 i.u./kg. of body wt.) given 4 hr. before slaughter				
14	D and S	1·87	0·3	1·90	15
15	D and S	1·8	0·1	1·9	5
20	D	9·0	11·0	20·0	55
20	S	12·0	13·0	25·0	52
21	D	21·0	7·0	28·0	25
21	S	20·0	5·0	25·0	20
22	D	8·9	51·7	60·6	85
22	S	3·37	6·2	9·57	64
23	D	74·0	66·0	140·0	47
23	S	30·0	74·6	104·6	71
30	D and S	8·1	3·6	11·7	31
				Average	43

RESULTS

Since it was impracticable in the present experiments to collect and examine the whole intestines and their contents, samples were taken from representative portions of the small intestine. Previous work showed this to be the site of the maximum absorption of vitamin A. The amount of food present in the intestinal tract differed from animal to animal, and the concentrations of vitamin A found in different animals varied. In two undosed animals, the amounts of vitamin A in the intestinal contents, wall and lymph were found to be negligible. Tables 1, 3 and 5 show the results obtained for calves, and Tables 2, 4 and 6 those for sheep.

Intestinal contents. Nearly complete hydrolysis was found to have occurred in the intestines of animals nos. 14, 15 and 43 4 hr. after administration of vitamin A esters, whereas in others the hydrolysis was only partial (Tables 1 and 2). When vitamin A alcohol was administered, the dose remained in the intestine in the free unesterified form (Table 1).

Table 3. *Concentrations of vitamin A alcohol and ester in the intestinal mucosa of calves*

Calf no.	Duodenum (D) or small intestine (S)	Vitamin A (i.u./g.) Alcohol fraction	Ester fraction	Total	Percentage of total vitamin A esterified
	Vitamin A ester (5000 i.u./kg. body wt.) given 4 hr. before slaughter				
55	D and S	7·9	12·6	20·5	61
56	D and S	1·1	3·9	5·0	78
43*	D	1·11	3·6	4·71	76
43	S	1·11	5·8	6·91	84
46*	D	2·12	18·0	20·12	89
46	S	2·7	6·0	8·7	69
47*	D	0·7	2·0	2·7	74
47	S	2·6	2·8	5·4	52
48*	D	1·2	5·5	6·7	82
48	S	0·6	1·3	1·9	68
				Average	**73**
	Vitamin A alcohol (5000 i.u./kg. body wt.) given 4 hr. before slaughter				
49	D	1·06	3·3	4·36	76
50	D	14·7	103·0	117·7	87
50	S	17·8	83·0	100·8	82
				Average	**82**
	Undosed animals				
52	D and S		0·12		
53	D and S		0·20		

* Newborn calves.

Table 4. *Concentrations of vitamin A alcohol and ester in the intestinal mucosa of sheep*

Sheep no.	Duodenum (D) or small intestine (S)	Vitamin A (i.u./g.) Alcohol fraction	Ester fraction	Total	Percentage of total vitamin A esterified
	Vitamin A ester (5000 i.u./kg. body wt.) given 4 hr. before slaughter				
14	D and S	0·56	0·86	1·46	60
15	D and S	0·32	0·56	0·88	64
20	D	3·64	7·26	10·9	67
20	S	1·84	1·96	3·80	52
21	D	5·9	1·71	7·61	22
21	S	6·3	2·96	9·26	32
22	D	3·57	4·60	8·17	56
22	S	1·41	4·83	6·24	77
23	D	3·29	3·79	7·08	53
23	S	8·5	38·6	47·1	82
30	D and S	0·6	0·51	1·11	46
				Average	**56**
	Vitamin A alcohol (5000 i.u./kg. body wt.) given 4 hr. before slaughter				
25	D	1·8	4·56	6·36	72
26	D	0·66	4·7	5·36	88
26	S	1·5	6·8	8·3	82
27	D	2·25	4·48	6·73	67
				Average	**77**

Intestinal wall. In general, after administration of vitamin A esters, most of the vitamin in the mucosa existed as ester, the percentage of the total vitamin A varying from 52 to 89 %, and from 22 to 82 %, in calves (Table 3) and in sheep (Table 4) respectively. When vitamin A alcohol was fed similar results were obtained, thus the percentage of the esterified form ranged from 76 to 87 % for calves (Table 3) and from 67 to 88 % for sheep (Table 4) respectively, showing that esterification had taken place.

Lymph and blood. After administration of either form of vitamin A, the lymph content was mostly (76–98 %) in the esterified form (Tables 5 and 6). In plasma from adult sheep, the average concentration of vitamin A alcohol before dosing was 98 i.u./100 ml., but only about 5 i.u./100 ml. were esterified (Table 7). After dosing, the rise was mainly in the ester fraction, but was only temporary. On the other hand, in newborn calves which usually have a low vitamin A alcohol level, a rise occurred after dosing in the vitamin A alcohol fraction as well (Table 7). The concentration of vitamin A alcohol in this instance increased from 20 to 55 i.u./100 ml. Preliminary experiments suggest that the increase was only temporary, as 24 hr. later it fell almost to the original levels.

DISCUSSION

Hydrolysis of vitamin A esters

Gray *et al.* (1940) found that vitamin A esters were hydrolysed in the intestines of adult rats. In the present work a similar observation has been made on ruminants. It has been suggested by Parrish, Wise & Hughes (1948) that the mechanism for the ab-

Table 5. *Concentrations of vitamin A alcohol and ester in the intestinal lymph of calves*

Calf no.	Vitamin A (i.u./ml.) Alcohol fraction	Ester fraction	Total	Percentage of total vitamin A esterified
Vitamin A ester (5000 i.u./kg. of body wt.) given 4 hr. before slaughter				
55	7·0	26·0	33·0	78
56	1·2	3·8	5·0	76
43*	4·14	15·4	19·54	79
46*	1·60	24·4	26·0	94
47*	0·90	8·0	8·9	90
48*	1·10	14·3	15·4	93
			Average	**85**
Vitamin A alcohol (5000 i.u./kg. of body wt.) given 4 hr. before slaughter				
49	0·87	5·98	6·85	87
50	5·30	72·7	78·0	93
			Average	**90**
Undosed animals				
52		0·18		
53		0·02		

* Newborn calves.

Table 6. *Concentrations of vitamin A alcohol and ester in the intestinal lymph of sheep*

Sheep no.	Vitamin A (i.u./ml.) Alcohol fraction	Ester fraction	Total	Percentage of total vitamin A esterified
Vitamin A ester (5000 i.u./kg. of body wt.) given 4 hr. before slaughter				
14	1·82	8·35	10·17	82
20	2·23	116·0	118·23	98
21	2·79	19·3	22·09	87
22	4·56	44·0	48·56	90
23	8·60	98·0	106·60	91
30	1·09	4·34	5·43	80
			Average	**88**
Vitamin A alcohol (5000 i.u./kg. of body wt.) given 4 hr. before slaughter				
25	2·9	38·1	41·0	93
26	1·68	14·9	16·58	40
27	1·42	39·8	41·22	97
			Average	**93**

Table 7. *Vitamin A alcohol and ester content in plasma before and after dosing*

(Dose administered: 5000 i.u./kg. of body wt.)

	Vitamin A in plasma (i.u./100 ml.)					
	Ester fraction			Alcohol fraction		
Animal no.	Before dosing	4 hr. after	Rise	Before dosing	4 hr. after	Rise
			Adult animals			
22	5	44	39	96	95	−1
23	5	49	44	132	139	7
27	7	48	41	120	127	7
1	2	109	107	45	42	−3
Average	**5**	**63**	**58**	**98**	**101**	**3**
			Young animals			
43	8	117	109	26	85	59
46	2	163	161	21	61	40
47	5	28	23	13	24	8
48	2	56	54	13	40	27
49	1	70	69	24	60	36
51	5	145	140	25	62	37
Average	**4**	**97**	**93**	**20**	**55**	**35**

sorption of vitamin A in newborn calves might differ from that occurring in adult animals. Recalling the claim of Howe (1924), that calves during the first few days of life may absorb unhydrolysed proteins, Parrish *et al.* (1948) discussed the possibility that the vitamin A esters of colostrum might also be absorbed without hydrolysis. In the present work, however, the absorption of vitamin A when mixed with 'reconstituted' milk (p. 261) was found to be essentially the same in newborn calves as in older animals (Tables 1, 3 and 5). Hydrolysis of vitamin A esters derived from colostrum was also observed to occur in the intestinal tract.

The possibility of the absorption of vitamin A esters

Although we have confirmed that considerable hydrolysis of vitamin A esters occurs in the intestinal tract, it does not follow that all the vitamin A esters have to be hydrolysed prior to absorption. Frazer (1946) has shown that complete hydrolysis of glycerol esters prior to absorption is not essential, and that a part of the ingested fat is absorbed as finely emulsified particles. If, in the present experiment, any vitamin A esters were absorbed, the percentage of esterified vitamin A in the intestinal mucosa should have been larger after administration of esters than after administration of vitamin A alcohol. This difference was not observed (Tables 3 and 4), and, so far, other workers have likewise been unable to obtain any evidence that esterified vitamin A is absorbed in its unaltered form.

Re-esterification of vitamin A

As most of the vitamin in the intestinal mucosa, after the feeding of vitamin A alcohol, was in the ester form, esterification must have taken place. Glover & Morton (1948) have suggested that vitamin A alcohol in the intestinal wall is esterified by the lipase concerned in the hydrolysis and resynthesis of fats, some of the fatty acids already present in the gut wall being utilized for this purpose. This theory is supported by the work of Gray, Hickmann & Brown (1940), who found that, when vitamin A esters containing unnatural fatty acids such as caproic acid were administered, the vitamin A in the liver was re-esterified with the fatty acids of natural glycerides. Thus the absence of vitamin A caproate in the liver gave further indirect evidence that vitamin A esters are hydrolysed before absorption.

The different roles of vitamin A alcohol and ester in the body

The results of the present work confirm our preliminary findings (Eden & Sellers, 1948) that, after the ingestion of vitamin A, most of it is found in the intestinal lymph in the esterified form. Goodwin & Gregory (1948) have also reported that under similar conditions 75% of the total vitamin A in the lymph was esterified. Thus it seems that after the re-esterification of vitamin A in the intestinal mucosa, the esters pass into the lymph, where they are probably incorporated into the chylomicrons and enter the general circulation as part of these fat particles. The hypothesis that the vitamin reaches the blood in esterified form is supported by the present experiments and by previous work on other species (Chevalier & Choron, 1938; Clausen, McCoord, Ryden & Breese, 1942; Hoch & Hoch, 1946). Thus, before dosing, the vitamin A in the blood is found mainly in the form of the alcohol, whereas after absorption, all the surplus of vitamin A in the blood is found in the form of the ester. This excess vitamin

A is removed generally within 24 hr. leaving the vitamin A content of the blood at the same level as before dosing.

In the present experiments, it was not possible to raise the level of vitamin A alcohol in the blood of adult animals by giving large doses of the vitamin. This suggests that each animal has a characteristic blood threshold of vitamin A alcohol. When the vitamin A content of the blood is at this level all the excess incoming vitamin A would remain esterified, whereas if the vitamin A content of the blood were below the threshold, some of the esters would be hydrolysed to raise the vitamin A alcohol content to the characteristic level. To test this hypothesis, we have carried out some preliminary experiments on the changes found in the blood of young calves after administering the vitamin. In calves the vitamin A content of the blood is usually much lower than in adults. A dose of the vitamin resulted in a temporary rise in the vitamin A alcohol of the blood, although some of the excess vitamin A was in the ester form. Such a rise of vitamin A alcohol following dosage has also been obtained by Thompson, Braude, Cowie, Ganguly & Kon (1949).

These studies on the relative distribution of the free (alcohol) and esterified form of vitamin A in the body show that, although the circulating vitamin A is mainly in the free form, the excess vitamin A in the body is esterified. As noted above, this is the form in which the excess vitamin A is present in the lymph and blood after absorption, and it is also the form in which it is stored in the liver (Gray, Hickmann & Brown, 1940; Gray & Cawley, 1942; Glover, Goodwin & Morton, 1947). All these tissues and body fluids, however, contain a small amount of free vitamin A, and the proportion increases as the total amount of vitamin A in the tissues decreases (Glover *et al.* 1947). Vitamin A alcohol may not be the form in which the vitamin exerts its physiological function in the body, but probably is a step nearer to this functional form than vitamin A esters, or the provitamins, such as carotene.

Note added 19 *November* 1949

Thompson, Ganguly & Kon (1949) have recently reported similar results on the absorption of vitamin A by rats and pigs.

SUMMARY

1. Calves and sheep were dosed with vitamin A alcohol or ester (5000 i.u./kg. of body weight) and slaughtered 4 hr. after dosing. The concentrations of vitamin A alcohol and of ester were determined in the blood before and after dosing, and, on slaughter, in the intestinal contents and wall and in the lymph.

2. Four hours after the administration of vitamin A esters, almost complete hydrolysis had occurred in the intestinal lumen of some animals, whereas in others hydrolysis was only partial.

3. Determinations made on the mucosa after dosing with vitamin A ester showed that the ester fraction contained 73 and 56 % of the total vitamin A in calves and sheep respectively. The values obtained after dosing with vitamin A alcohol were 82 and 77 % for the two species, showing that esterification had taken place.

4. After passing across the intestinal wall the absorbed vitamin A apparently remained esterified, as the rise in the vitamin content of the intestinal lymph following administration of either form was nearly all in the ester fraction. In the normal adult blood also the increase after administration of vitamin A was mainly in the ester form.

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The Metabolism of Sulphapyridine Containing Radioactive Sulphur in the Rabbit

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This paper records a further stage in the attempt to draw up a balance sheet for the fate of a single dose of sulphapyridine in the rabbit. Bray, Neale & Thorpe (1946) found that a considerable proportion of a dose of sulphapyridine could not be accounted for in rabbit urine by methods of estimation based upon the diazo reaction, especially at higher dose levels (0·5–1·0 g./kg.). We were unable significantly to improve the recovery by analysis of the faeces or contents of the alimentary canal. An increased excretion of inorganic sulphate in approximately one-third of the animals roughly corresponding to the missing sulphapyridine suggested the possibility of complete catabolism, but since the increased excretion was almost within the base-line range we did not feel justified in accepting this interpretation without further evidence. The diazo method upon which we depended for estimation of the metabolites would not have revealed the presence of a metabolite in which the amino group had been modified in such a way that it could not be liberated by ordinary hydrolysis procedures. The estimation of the total sulphur of the urine would include such a metabolite, but since the expected amount of this metabolite was of the same order as that of the neutral sulphur normally excreted by the rabbit, it would be difficult to obtain conclusive information as to the extent of the excretion of a nondiazotizable metabolite owing to fluctuations in the neutral sulphur normally excreted.

In order clearly to distinguish between the excretion of the normal neutral sulphur and that due to sulphapyridine we have carried out experiments with the drug labelled with the radioactive isotope ^{35}S. These experiments indicate that there is no complete catabolism resulting in the elimination of the sulphur as inorganic sulphate. The consistently higher recovery of the drug as estimated by radioactive assay than by the diazo method suggests the formation of a metabolite in which the amino group of sulphapyridine has been eliminated, although we have so far failed to isolate such a compound. There is no appreciable retention of the drug in the tissues.

EXPERIMENTAL

Material and Methods

Preparation of radioactive sulphapyridine. ^{35}S was received in the form of a solution of $Na_2{}^{35}S$ in NaOH (0·2 ml.). The activity of the solution on despatch was stated to be 212 μc. The method of synthesis is summarized as follows, an asterisk indicating the presence of ^{35}S:

$$Na_2S^* \xrightarrow{H_2O_2} Na_2S^*O_4 \xrightarrow{H_3PO_4} H_2S^*O_4 \xrightarrow{C_6H_5NH_2}$$
$$\rightarrow H_2N.C_6H_4.S^*O_3H \xrightarrow{NaOH} H_2N.C_6H_4.S^*O_3Na \xrightarrow{Ac_2O}$$
$$\rightarrow CH_3CO.HN.C_6H_4.S^*O_3Na \xrightarrow{PCl_5} CH_3CO.HN.C_6H_4.S^*O_2Cl \xrightarrow{C_5H_4N.NH_2}$$
$$\rightarrow CH_3CO.HN.C_6H_4.S^*O_2NH.C_5H_4N \xrightarrow{HCl/EtOH} H_2N.C_6H_4.S^*O_2NH.C_5H_4N.$$

Radioactive sodium sulphate. The NaOH containing Na_2S^* was added to a filtered solution of $Na_2S.9H_2O$ (10 g.) in water (50 ml.); with washings the total volume was 120 ml. This was transferred to a large covered beaker and a mixture (160 ml.) of equal volumes of 2 N-NH_4OH and H_2O_2 (40 vol.) added. Heat was evolved. After 15 min. the mixture was heated gently until the evolution of O_2 ceased. The solution was then carefully evaporated to dryness. Dry weight 6·6 g.

Radioactive sulphuric acid. The $Na_2S^*O_4$ was mixed with anhydrous Na_2SO_4 and the mixture (32·45 g.) finely ground. H_3PO_4 (18 ml. of 100%) was added and the mixture distilled over a naked flame. Considerable care was necessary to avoid frothing during the early stages of heating. Distillation was continued until the contents of the flask were at a dull red heat. A small amount of water was removed from the distillate by redistillation. Yield 19·3 g. (86% of theory). (We had expected to receive ^{35}S in the form of $BaSO_4$. H_2SO_4 can be prepared from $BaSO_4$ by fusing with excess Na_2CO_3 and K_2CO_3. After removal of $BaCO_3$ and neutralization of excess alkali carbonates with H_2SO_4 the dried mixed sulphates are distilled with H_3PO_4 as above.)

Radioactive sulphanilic acid. The method used was essentially that of Erdmann (1894). The above $H_2S^*O_4$ was washed with conc. H_2SO_4 (15 ml.) into a small bolthead flask and aniline (55 ml.) added. The mixture was heated at 210–230° in an oil bath for 3·5 hr., cooled and dissolved in Na_2CO_3 solution (750 ml., 50 g./l.). The blue solution was

decolorized with charcoal and acidified with conc. HCl. Radioactive sulphanilic acid separated and was collected and washed with water. A second crop was obtained from the filtrate. Total yield 60 g. The acid was then converted to the Na salt by the addition of 2 N-NaOH. The solution was evaporated to dryness under reduced pressure and dried for 48 hr. *in vacuo* over P_2O_5.

Radioactive acetamidosulphanilyl chloride. This was prepared from the Na sulphanilate by the method of Schroeter (1906). Yield 37·7 g. The mother liquors on keeping deposited radioactive sulphanilic acid which was separated and treated again.

Radioactive sulphapyridine. The condensation of the acetamidosulphanilyl chloride with 2-aminopyridine and subsequent deacetylation was carried out according to Winterbottom (1940). Total yield of radioactive sulphapyridine 12·4 g. including 3 g. from reworking of the residues from the various stages. Melting point alone or mixed with sulphapyridine (m.p. 189°), 189°. (Found: N, 16·80 %. Calc. for $C_{11}H_{11}O_2N_3S$: N, 16·87 %.) The intensity of colour in the diazo reaction was the same as that of the standard sulphapyridine. The radioactivity of this material, determined 5 weeks after the assay of the original ^{35}S, was such that it gave, on our apparatus, 15,300 counts/min./g. sulphapyridine or 16·5 counts/min./mg. $BaSO_4$.

Animals, diet and dosage. Rabbits weighing 2 kg., which had not previously been used for experimental purposes were used and maintained for a preliminary period and throughout the experiment on the constant diet previously described (Bray, Ryman & Thorpe, 1947). Sulphapyridine was administered by stomach tube as a suspension in water.

Ashing of organic material for estimation of ^{35}S. The S in all organic substances, and tissues, etc., was converted to $BaSO_4$ by the modification of Pirie's (1932) ashing process used by Boursnell, Francis & Wormall (1946). Sulphapyridine treated in this way gave a quantitative yield of $BaSO_4$ within 0·7 %. The weight of $BaSO_4$ was determined gravimetrically on a separate similar sample in each case.

Preparation of samples for ^{35}S *assay. Urine.* Inorganic and total sulphate were precipitated from urine as in the Folin (1905–6) method, isolated by centrifuging and then treated by the ashing process referred to above.

Total sulphur. Urine. 20 or 25 ml. were evaporated to 5–10 ml. on a water bath with a few drops of conc. HNO_3. The concentrate was ashed and converted to $BaSO_4$ as above. Several similar samples gave results within ±1 % and with added sulphapyridine the results were within −2 % of the theoretical.

Faeces. Specimens (2 g.) were treated with 5 ml. of conc. HNO_3, warmed gently till frothing ceased and then ashed and converted to $BaSO_4$.

Tissues. These were treated in the same way as faeces. If necessary the ashing process was repeated. The bulk of the $BaSO_4$ precipitate (the ^{35}S assay required approx. 25 mg. of $BaSO_4$) was increased when necessary by adding 0·5 ml. of a solution of K_2SO_4 (37·28 g./l.) to the solutions of the ashed specimens before precipitation. In this way the weight of the $BaSO_4$ was increased by 25 mg.

Assay of ^{35}S. The ^{35}S was determined by the method described by Banks (1946).

Estimation of diazotizable material. The method of Bratton & Marshall (1939) was again used. The quantities of reagents were shown to be sufficient to react with all the diazotizable material in rabbit urine under the conditions used. The maximum error observed in determining known amounts of sulphapyridine added to normal or metabolic (sulphapyridine) urines was ±5 %. There was no significant difference in estimations of sulphapyridine in hydrolysed or unhydrolysed urine, so that glucuronic acid liberated from hydroxysulphapyridine glucuronide on hydrolysis does not appear to interfere. Kirch & Bergeim (1943) reported that ascorbic acid and other reducing agents interfered with diazo estimations.

The diazotizable material in faeces was estimated as previously (Bray *et al.* 1946). Tissues were homogenized with up to 10 parts water to 1 part tissue and treated as blood in the Bratton & Marshall (1939) method.

Sampling and collecting errors. Acetylsulphapyridine (about 300 mg.) usually separated from the urine of rabbits heavily dosed with 2–3 g. of sulphapyridine, even when the volume of urine was large. The error in sampling such urines determined on many samples of the same urine was found to be ±1·1 %.

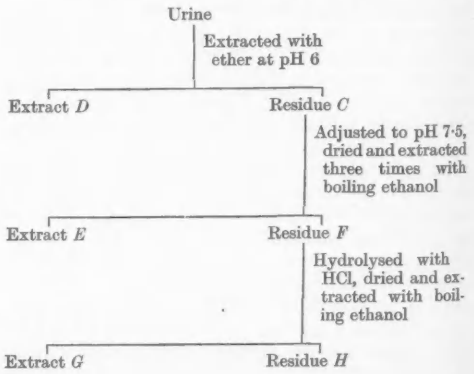
In spite of daily washing of the metal funnels of the metabolism cages when the day's urine is collected, films of 'phosphate' tend to adhere to the funnels. These have to be removed at intervals with acid, and occasionally a positive diazo reaction can be obtained from the acid extract of the deposit. The significance of this possible error was tested by collecting, through a funnel which had been allowed to accumulate an extensive film of 'phosphate' from normal urine, the 24 hr. urine from a rabbit dosed with 2 g. of sulphapyridine. The film was then dissolved in acid and the solution hydrolysed. It yielded 12·6 mg. of diazotisable materia l(calc. as sulphapyridine), i.e. 0·6 % of the dose. With a freshly cleaned funnel only 6 mg. could be detected.

Fractionation of urine

The urine fractions referred to in Table 3 were obtained as follows: (*A*) Urine was treated with a saturated solution of basic lead acetate in the usual way. The precipitate was removed and the filtrate treated with hydrogen sulphide to remove lead. (*B*) The precipitate from *A* was suspended in water and lead removed as lead sulphide.

Fractions *C* to *H* were obtained as shown in Table 1.

Table 1. *Fractionation of urine*


Urine
Extracted with ether at pH 6
Extract D
Residue C
Adjusted to pH 7·5, dried and extracted three times with boiling ethanol
Extract E
Residue F
Hydrolysed with HCl, dried and extracted with boiling ethanol
Extract G
Residue H

RESULTS

Table 2 shows the results of diazo and ^{35}S estimations on the excreta and tissues of four rabbits which had each received doses of radioactive sulphapyridine.

bolite. The preparation of urine fractions *A* to *H* has already been described. The distribution of metabolites in these fractions is given in Table 3. In the absence of any obvious and consistent separation in the distribution of radioactive and diazotizable

Table 2. *Recovery of sulphapyridine in the rabbit estimated by ^{35}S assay and diazo method expressed as percentage of dose*

Exp. no.	1		2		3		4	
Rabbit no.	203		205		211		210	
Dose (g.)	2·0		2·5		2·2		3·0	
Urine	^{35}S	Diazo	^{35}S*	Diazo	^{35}S*	Diazo	^{35}S	Diazo
Inorg. SO_4 1st day	0†	—	0	—	—	—	—	—
Inorg. SO_4 2nd day	0	—	0	—	—	—	—	—
Total SO_4 1st day	0	—	0	—	0	—	—	—
Total SO_4 2nd day	0	—	0	—	—	—	—	—
Total S 1st day	43·2	33·0	25·7	24·0	79·0	61·8	61·4	49·9
Total S 2nd day	10·5	8·4	30·4	26·8	—	(4·1)	19·8	15·0
Total S 3rd day	—	—	33·5	21·6	—	(0·8)	—	—
Bladder urine at death	—	—	0·5	0·4	—	—	—	—
Faeces								
1st day	6·5	4·0	2·6	2·4	10·6	4·0	—	—
2nd day	6·1	6·0	0·8	0·7	4·7	1·0	—	—
3rd day	—	—	—	—	3·5	0	—	—
Alimentary canal‡								
Stomach contents	—	—	1·7	0	0	0	—	—
Small intestine contents	—	—	—	0	0	0	—	—
Caecal contents	—	0	2·4	0·8	0	0	—	—
Colon contents	—	—	0·3	0·2	0	0	—	—
Bile	0	0	—	—	—	—	—	—
Tissues								
Blood	0	0	—	—	—	—	—	—
Brain	—	—	0	0	—	—	—	—
Gut wall	—	—	0	0·1	—	—	—	—
Hair	0	0	—	—	—	—	—	—
Heart	—	—	0	0	—	—	—	—
Kidney	0	Tr.	1·8	0·2	—	—	—	—
Liver	0	0	0	0·1	0·0	0·1	—	—
Muscle	0	0	0	0	—	—	—	—
Skin	0	0	—	—	—	—	—	—
Spleen	0	0	—	—	—	—	—	—
Recovery	66·3	51·4	99·7	77·3	97·8	66·9§ (71·8)	81·2	64·9

* Owing to some doubt as to accuracy of the radioactivity counts of the standard preparations, the results in these two experiments have been calculated from the radioactivity of the nonsulphate fraction of the urinary S compounds which is composed almost entirely of sulphapyridine or its metabolites.

† Values of 0 for ^{35}S assays indicate that the count of the samples was not significantly greater than the background count of the apparatus.

‡ The contents of the alimentary tract were removed 72 hr. after the administration of the sulphapyridine.

§ This total is comparable with that obtained from ^{35}S determinations. That in parenthesis is the true total including the second and third day total S values (given in parenthesis) which were not estimated by the ^{35}S method.

In Exps. 1–3 the rabbit was killed at the end of the third 24 hr. period after the dose, when urinary excretion of the dose was virtually complete. Exps. 1 and 2 were designed to include a large number of tissues, together with urine and faeces. Since these experiments gave no indication of storage in the tissues, a more detailed examination of intestinal contents was made in Exp. 3. In Exp. 4 an attempt was made to fractionate the urine with a view to directing the search for a nondiazotizable metabolite. The preparation of urine fractions *A* to *H* has already been described. The distribution of metabolites in these fractions is given in Table 3. In the absence of any obvious and consistent separation in the distribution of radioactive and diazotizable material, it seemed that fraction *D*, the ether extract, would be the most profitable one to examine for a nondiazotizable metabolite.

Search for a nondiazotizable metabolite. Two attempts were made to detect such a metabolite in the solid material obtained by extraction with ether at pH 6 for 48 hr. At this pH very little ether-soluble material is extracted from normal rabbit urine. (1) The ether-soluble material from 24 hr. urines of forty rabbits dosed with a total of 90 g. of sulpha-

Table 3. *Distribution of sulphapyridine and metabolites in urine fractions expressed as percentage of dose*

Exp. no. ...	3		4		4	
	1st day		1st day		2nd day	
Fraction*	^{35}S	Diazo	^{35}S	Diazo	^{35}S	Diazo
Total S	79·0	61·8	61·4	49·9	19·8	15·0
A	13·9	10·8	8·3	9·7	—	—
B	—	—	32·6	25·2	—	—
C	25·7	9·4	23·3	16·4	6·3	4·2
D	—	—	39·3	31·6	9·2	11·2
E	—	—	9·5	6·2	1·8	1·5
F	—	—	—	—	5·2	2·3
G	—	—	2·9	2·4	—	—
H	—	—	5·2	3·8	—	—

* The preparation of these fractions has been described in the text.

pyridine was dissolved in 50 % aqueous ethanol, decolorized with a small amount of charcoal and progressively concentrated to yield successive crops of crystals. These fractions were mixtures of sulphapyridine and acetylsulphapyridine, which, when hydrolysed, gave only sulphapyridine, m.p. 189°, alone or mixed with an authentic specimen. (2) The ether-soluble material from urine corresponding to a dosage of 60 g. of sulphapyridine was hydrolysed by boiling with 250 ml. of 2N-sodium hydroxide. Crude sulphapyridine was precipitated by neutralization with hydrochloric acid and was separated. The filtrate was evaporated to dryness and extracted three times with boiling absolute ethanol, which removed a small amount (0·5 ml.) of phenolic material (S free). The ethanol insoluble material was almost entirely sodium chloride. The crude sulphapyridine was fractionally crystallized. After separation of large amounts of sulphapyridine, m.p. 189°, a final fraction (1 g.), m.p. 182–185° (mixed melting point with sulphapyridine 185–189°), was obtained which gave colours with ferric chloride and with acid nitrite similar to those given by hydroxysulphapyridine (Bray *et al.* 1946). All the other sulphapyridine fractions gave the yellow colour with nitrite, but no ferric chloride colour, although the impurity was not sufficient to depress the melting point or affect quantitative diazo estimation. All fractions identified as sulphapyridine gave the expected values by diazo estimation. No other metabolites were detected and it was concluded that the crude sulphapyridine consisted of sulphapyridine mixed with very small amounts of hydroxysulphapyridine.

DISCUSSION

It is clear from Exps. 1–3 that no significant amount of ^{35}S is excreted in either the inorganic or total sulphate of urine, since in every case the radioactivity of the barium sulphate from these fractions was not significantly greater than the background count of the apparatus corresponding to less than 0·2 % of the dose. Thus there is no support for the previous suggestion (Bray *et al.* 1946) that some sulphapyridine is completely catabolized. The experiments also indicate that there is no appreciable storage of the drug in the tissues. Only the kidney in Exp. 2 contained significant amounts of ^{35}S and this is, perhaps, not surprising in view of the lag in excretion of the drug by this animal. It is rare to find so high an excretion on the third day. The presence of ^{35}S in the alimentary canal after the third day in this experiment suggests that this animal absorbed the drug more slowly than did the others. Kinnunen (1946) also was unable to find evidence of storage of sulphapyridine. He noted that after administration of aneurin, together with sulphapyridine, there was a decreased excretion of diazotizable material in urine but no storage in the tissues.

The values obtained by the ^{35}S assays were almost always greater than those by diazo estimations. When the urine was fractionated as in Exp. 4, these differences persisted, but there was no indication of the separation of a fraction with a much higher ^{35}S/diazo ratio. With regard to the actual average difference of about 25 % between the ^{35}S and diazo values for material excreted in urine, it has been shown that the errors in the chemical procedures of the two methods are of the order of -2 % and ± 5 %, respectively, so that the maximum discrepancy which should have been observed would be of the order of -7 %, leaving nearly 20 % to be accounted for. It was this which prompted our search for a nondiazotizable metabolite. The difficulty of separating such a metabolite from sulphapyridine, as indicated by the similarity of the ^{35}S/diazo ratios of the urine fractions, makes it probable that this metabolite, if it exists, has a structure close to that of sulphapyridine, possibly only the amino group being modified. The attempts at isolation of this metabolite have so far been unsuccessful.

SUMMARY

1. Sulphapyridine containing ^{35}S has been synthesized.

2. Metabolic experiments with this compound in the rabbit have shown that there is no complete catabolism to inorganic sulphate and no significant retention of the drug in the tissues after 3 days.

3. Differences between the values obtained by assays of ^{35}S and diazotizable material suggested the formation of a metabolite which does not diazotize or couple normally but this has not yet been isolated.

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The Fate of Certain Organic Acids and Amides in the Rabbit

10. THE APPLICATION OF PAPER CHROMATOGRAPHY TO METABOLIC STUDIES OF HYDROXYBENZOIC ACIDS AND AMIDES

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Metabolic processes resulting in hydroxylation of aromatic compounds are well known. The proportion of a given compound so changed varies however with the structure and the consequent metabolic alternatives. If the compound already contains conjugable groups or readily convertible 'potential centres for conjugation' (Bray, Ryman & Thorpe, 1948) the phenols obtainable by hydroxylation may only be formed in small amount. In the past it has often not been possible to identify and determine very small quantities of such phenolic compounds in urines containing normal amounts of other phenols. We have now shown, however, that the method of paper chromatography originally developed for identification of small amounts of amino-acids by Consden, Gordon & Martin (1944) can usefully be adapted to the identification of phenolic compounds. This paper shows how the method has enabled us to confirm and to extend some earlier observations on the hydroxylation and glycine conjugation of the hydroxybenzoic acids and their amides.

METHODS

Paper

Whatman filter paper no. 4 was used.

Solvents

(All percentages are v/v.) The two main series of developing solvents used consisted of benzene–acetic acid–water and $CHCl_3$–acetic acid–water. By varying the relative proportions of the constituents, R_F values could be varied within wide limits. By increasing the amount of acetic acid (formic acid was equally effective) the R_F value of a compound was increased. By these means R_F values could be adjusted so as to give maximum separation and bring compounds into suitable positions on the chromatogram. The benzene series, especially benzene (40%), acetic acid (40%), water (20%), was suitable for the majority of the compounds. The $CHCl_3$ series, e.g. $CHCl_3$ (50%), acetic acid (25%), water (25%), was used for separating compounds having low R_F values in the benzene series. *n*-Butanol–acetic acid–water mixtures gave similar results to the $CHCl_3$ series. The acetic or formic acid in these mixtures greatly reduced 'trailing'. These solvents have proved suitable for detection of aromatic monohydroxy acids and amides and

dihydroxy compounds, but we have not yet found a solvent mixture suitable for phenols, cresols and xylenols.

Apparatus and procedure

An ascending method essentially similar to that of Williams & Kirby (1948) was used. Cylinders of the paper, usually about 33 cm. high, were made from sheets by stapling so that the edges did not overlap. Spots of the required solutions containing 1–5 μg. of the substance were applied to the paper so as to lie about 2·5 cm. from the lower edge of the cylinder and dried. The cylinder was placed in a Petri dish containing the non-aqueous phase of the solvent mixture, the whole standing in a filtrate jar, the bottom of which was covered with the aqueous phase of the mixture. The jar was covered with a glass food cover. A run of 3–5 hr. was sufficient for hydroxybenzoic acids, but for slower moving compounds, e.g. dihydroxy compounds and *m*- and *p*-hydroxyhippuric acids, a longer time, up to 15 hr., was required, evaporation at the solvent front taking place. The positions occupied by the compounds after development were revealed by spraying, after drying, with an appropriate detecting reagent.

Detecting reagents

(1) *Diazotized* p-*nitraniline.* (*a*) *p*-Nitraniline, 0·3% in 8% (w/v) HCl (25 ml.) and $NaNO_2$ (5% w/v) (1·5 ml.) mixed immediately before spraying. (*b*) Na_2CO_3 (20% w/v). The two solutions were used successively.

(2) *Diazotized sulphanilic acid.* This was prepared and used as above, sulphanilic acid (0·3% in 8% w/v HCl), replacing *p*-nitraniline.

(3) 2:6-*Dichloroquinone chloroimide.* (*a*) 2:6-Dichloroquinone chloroimide (0·1% w/v in absolute ethanol) freshly prepared; (*b*) veronal or phosphate buffer, pH 10. The two solutions were sprayed successively.

(4) *Ammoniacal silver nitrate.* 0·1 N-$AgNO_3$ (25 ml.) and NH_4OH (8 ml., sp.gr. 0·880) made up to 50 ml. with water.

(5) *Ferric chloride.* 0·2% (w/v) in water.

The diazo reagents are generally suitable for detection of the hydroxy acids and the $AgNO_3$ reagent for the dihydroxy compounds. The quinone reagent tends to be hypersensitive making identification in urine extracts difficult. The $FeCl_3$ reaction is not very sensitive when applied on paper and the colours are not always the same as those in the test tube. The responses of the phenolic compounds studied to some of these reagents are given in Table 1.

R_F values

The R_F values of a number of hydroxy acids, amides and dihydroxy compounds are given in Tables 1 and 2. It will be seen that by selecting appropriate solvents and detecting reagents, most of these compounds can be identified without ambiguity. It should be noted, however, that the solvent mixtures are liable to change in composition, on exposure to the atmospheric conditions in the apparatus, sufficiently to cause appreciable variation in R_F values. Changes in atmospheric temperature had a similar effect, but we did not determine whether this was related to accompanying changes in humidity. For our purpose, however, these variations were of little importance since reference compounds were always run on the chromatograms side by side with the unknowns.

Table 1. *Colour reactions and R_F values of some phenolic compounds*

(2:6-Dichloroquinone chloroimide gives a blue colour with all the compounds except salicyluric acid, with which it gives an intense blue-green colour. Solvent mixture: *A*, benzene 40, glacial acetic acid 50, water 10% (all % v/v); *B*, benzene 40, glacial acetic acid 40, water 20%; *C*, benzene 40, glacial acetic acid 30, water 30%.)

Compound	Colour with diazotized		Reaction with ammoniacal $AgNO_3$	R_F value in solvent mixture				
				A	*B*			*C*
	p-Nitraniline	Sulphanilic acid		(15 hr.)	(15 hr.)	(6 hr.)	(3 hr.)	(15 hr.)
Salicylic acid	Pale yellow	Yellow	–	.	1·0	1·0	1·0	.
Saligenin	Mauve	.	–	.	0·95	0·8	.	.
p-Hydroxycoumaric acid	Blue	.	–	.	0·95	.	.	.
Salicylamide	Orange	.	–	.	.	.	0·9	.
m-Hydroxybenzoic acid	Red	Yellow	–	.	0·9	0·6	0·5	.
p-Hydroxybenzoic acid	Red	Yellow	–	.	0·9	0·6	0·5	.
p-Hydroxyphenylacetic acid	Blue	Red	–	.	0·85	0·55	0·5	.
Salicyluric acid	Orange	Yellow	–	.	0·75	0·5	0·5	.
2:4-Dihydroxybenzoic acid	Brown	.	–	.	0·7	.	.	.
m-Hydroxybenzamide	Red	.	–	.	.	.	0·15	.
p-Hydroxybenzamide	Red	.	–	.	.	.	0·1	.
m-Hydroxyhippuric acid	Red	Yellow	–	.	0·05	0·0	0·0	.
p-Hydroxyhippuric acid	Pale red	Yellow	–	.	0·05	0·0	0·0	.
2:3-Dihydroxytoluene	Pale brown	.	+	0·8–1·0	0·9–1·0	.	.	.
Catechol	Pale brown	.	+	.	0·9–1·0	.	0·75	.
2:3-Dihydroxybenzoic acid	Pale brown	.	+	0·8–1·0	0·9–1·0	.	0·75	0·7
2:3-Dihydroxybenzamide	Pale brown	.	+	0·8–1·0	0·9–1·0	.	0·65	0·4
2:5-Dihydroxytoluene	Pale brown	.	+	0·8–1·0	0·7	.	.	.
2:5-Dihydroxybenzoic acid	Pale brown	Pale yellow	+	0·8	0·5	.	0·35	0·15
Quinol	Pale brown	.	+	.	0·3	.	0·25	.
2:5-Dihydroxybenzamide	Pale brown	.	+	0·65	0·25	.	0·2	0·0–0·15
3:4-Dihydroxytoluene	Pale brown	.	+	.	0·2	.	0·0–0·2	0·0–0·15
3:4-Dihydroxybenzoic acid	Pale brown	.	+	0·45	0·2	.	0·0–0·2	0·0–0·15
3:4-Dihydroxybenzamide	Pale brown	.	+	0·25	0·05	.	0·0–0·2	0·0–0·15

Table 2. *R_F values of some phenolic compounds*

(Solvent mixture: *D*, *n*-butanol 50, glacial acetic acid 5, water 45% (all %, v/v); *E*, aqueous benzene containing 0·1% acetic acid; *F*, $CHCl_3$ 50, glacial acetic acid 25, water 25%; *G*, benzene 50, glacial acetic acid 5, water 45%.)

Compound	R_F value in solvent mixture			
	D (6 hr.)	*E* (6 hr.)	*F* (3 hr.)	*G* (15 hr.)
Salicylic acid	1·0	0·0–0·4	.	.
Vanillic acid	.	.	.	0·65
m-Hydroxybenzoic acid	0·9	.	0·7	0·07
p-Hydroxybenzoic acid	0·9	.	0·7	0·07
m-Hydroxyhippuric acid	0·7	.	0·3	.
p-Hydroxyhippuric acid	0·7	.	.	.

Preparation of urine extracts

Known fractions of 24 hr. urines (about one-tenth was usually convenient, corresponding to about 50 mg. of administered compound) were acidified (with 0·2 vol. of 2N-H_2SO_4) and continuously extracted with ether for 6 hr. to extract unconjugated phenols. The acid content of the urine was then adjusted to 2 N and the urine was hydrolysed by boiling under reflux for 1 hr. and again continuously extracted with ether for 20 hr. to remove phenols originally conjugated.

The residues after removal of solvent from the two extracts were separately taken up in 5 ml. of acetone. Extracts of normal urine were used in conjunction with reference compounds to provide standards for identification or estimations.

Quantitative estimation

The method can be rendered roughly quantitative by comparing the size and intensity of the spots given by a known volume of unknown and standard solutions. This was most conveniently achieved by using a suitably graded series of standard spots of the reference compound alternating with those of the unknown. Reasonably accurate estimates, within 15% of the actual amount estimated, were obtained if the amount of compound used was between 1 and 5 μg. Measurement of the area of the spots as suggested by Fisher also gave satisfactory results (Fisher, Parsons & Morrison, 1948; Fisher & Holmes, 1949).

Animals, diet and dosage

Rabbits were fed and dosed as in previous investigations (Bray, Ryman & Thorpe, 1947).

RESULTS

Phenolic constituents of normal rabbit urine

Several phenolic compounds are known to occur in normal urine and may be revealed on the chromatograms. These normal constituents must be allowed for in the interpretation of chromatograms from metabolic urines. Most conspicuous in urines from rabbits on our standard pellet diet are *p*-hydroxybenzoic and *p*-hydroxyphenylacetic acids which occur free, and hydroquinone which is conjugated. So far we have not been able to distinguish between *m*- and *p*-hydroxybenzoic acids on paper chromatograms of urine extracts. It is therefore possible that *m*-hydroxybenzoic acid may also be a normal constituent of urine (cf. Lederer & Polonsky, 1948). We have assumed that the spot observed on the chromatograms is due mainly to the para isomer since this is a well known constituent of vertebrate urines (Lederer & Polonsky, 1948). Normal rabbit urine was also found to contain small amounts of catechol and 3:4-dihydroxybenzoic acid (both conjugated) and up to the present we have identified traces of 2:3- and 2:5-dihydroxybenzoic acids (both conjugated), vanillic acid and *p*-coumaric acid (both free; cf. Lederer, 1949). We did not detect 3:4-dihydroxytoluene, which we have shown to be a metabolite of *p*-cresol (Bray, Thorpe & White, 1950). It has already been mentioned that we have not yet found a solvent mixture suitable for the detection of phenol and cresols.

Hydroxylation of the hydroxybenzoic acids and amides

Accounts of investigations of these compounds by ordinary chemical methods have been published (Bray *et al.* 1947; Bray, Ryman & Thorpe, 1948), but although the main metabolic pathways of these six compounds were traced some details, relating to the hydroxylation products, could not be satisfactorily elucidated. The hydroxylation products which have since been detected in ether extracts of the urines by means of paper chromatograms are shown in Table 3.

Both the dihydroxy compounds which can be formed from salicylic acid and salicylamide by *o*- or *p*-hydroxylation (Bray, Lake, Neale, Thorpe & Wood, 1948) have been detected in rabbit urine after administration of the *o*-hydroxy compounds. All three possible dihydroxy compounds have been found in *m*-hydroxybenzamide urine, although the 2:3 compound was not detected in *m*-hydroxybenzoic acid urine. Previously, using ordinary chemical methods, we had been able to isolate only 2:5 compounds, gentisic acid or its amide, as metabolites of the *o*-hydroxy compounds. There was indirect evidence that gentisic acid, or amide, was formed from *m*-hydroxybenzamide in that a nitrogen-containing complex was separated from the urine which yielded hydroquinone on dry distillation (Bray, Ryman & Thorpe, 1948, pp. 564–5). Reinvestigation of this complex on the paper chromatogram has shown that it contained gentisic acid, its amide, 2:3-dihydroxybenzoic acid and its amide.

The present investigation has also revealed the true extent of hydroxylation of salicylic acid and salicylamide. Since a hydroxybenzoic acid contains two conjugable groups whereas its amide contains one and one potential centre for conjugation, which is not readily convertible, it would be expected that the amide would be hydroxylated to a greater extent

Table 3. *Hydroxylation products of hydroxybenzoic acids and amides in the rabbit detected by paper chromatography*

(The symbols + + indicate up to 3% of the dose, + up to 1%, − not identified.)

	Metabolites identified											
	Dihydroxybenzoic acids						Dihydroxybenzamides					
	2:3		2:5		3:4		2:3		2:5		3:4	
	Free	Conjugated	Free	Conjugated	Free	Conjugated	Free	Conjugated	Free	Conjugated	Free	Conjugated
o-Hydroxybenzoic acid	+	−	+ +	+	.	.	.	.	.	.	.	.
o-Hydroxybenzamide	−	+	−	+	.	.	−	+	+	+ +	.	.
m-Hydroxybenzoic acid	−	−	+ +	−	+ +	+	.	.	.	.	.	.
m-Hydroxybenzamide	−	−	−	+	−	+	−	+	+	+ +	−	+
p-Hydroxybenzoic acid	.	.	.	.	+ +	+	.	.	.	.	.	.
p-Hydroxybenzamide	.	.	.	.	−	+	.	.	.	.	+	+ +

than the acid. In the earlier investigations this appeared to be true for the *m*- and *p*-isomers, but the isolation of gentisic acid in greater amount from salicylate urine than from salicylamide urine suggested that the generalization did not apply to the *o*-isomers. It is now clear, however, from paper chromatograms that the total extent of hydroxylation of the amide is greater than that of the acid so that the *o*-isomers do conform with the generalization. The original difficulty in isolating hydroxylation products from salicylamide would seem to be due to that of separating four compounds having very similar properties.

Glycine conjugation of o- *and* m-*hydroxybenzoic acids*

It is well established that salicyluric acid is a metabolite of salicylic acid in man (Quick, 1933; Kapp & Coburn, 1942). In our earlier investigation we were unable to isolate it from rabbit salicylate urine, although we had no difficulty in obtaining it from human salicylate urine (Bray, Ryman & Thorpe, 1948). We have now shown by paper chromatogram that salicyluric acid is excreted by the rabbit to an extent of about 5% of the dose.

Owing to failure to isolate *m*-hydroxyhippuric acid we had also been led to conclude that conjugation of *m*-hydroxybenzoic acid with glycine did not occur in the rabbit. When, however, we examined paper chromatograms of ether extracts of *m*-hydroxybenzoic acid urines we observed a spot in a position which, by analogy with the *p*-isomer, *m*-hydroxyhippuric acid would be expected to occupy. (At that time we had no *m*-hydroxyhippuric acid to use as a reference compound.) Guided by chromatograms we were able to isolate the substance responsible, separating it from *m*-hydroxybenzoic acid by means of cold ether in which the latter is more readily soluble. This was found more satisfactory than the extraction with boiling toluene used previously.

The substance formed colourless needles, m.p. 185°. On hydrolysis with 5N-HCl for 6 hr. *m*-hydroxybenzoic acid and glycine, both identified by paper chromatogram, were formed. It was noted that the compound did not depress the melting point of hippuric acid, m.p. 187°. (Found: C, 55·4; H, 4·8; N, 7·5%. Equiv. by titration 193. Calc. for *m*-hydroxyhippuric acid, $C_9H_9O_4N$: C, 55·4; H, 4·6; N, 7·2%. Equiv., 195. Calc. for hippuric acid, $C_9H_9O_3N$: C, 60·2; H, 5·0; N, 7·8%. Equiv., 179.) It seems probable that our original failure to isolate *m*-hydroxyhippuric acid was largely due to confusion with hippuric acid which has practically the same melting point which is not depressed by admixture; in the absence of a chromatogram, melting point had been the chief guide in isolation. Recently, through the kind co-operation of Prof. F. Smith and Prof. Homer Smith, we received a specimen of *m*-hydroxyhippuric acid which was in every respect identical with our specimen.

Hydroxylation by rabbit-liver slices

It was noted in an earlier paper (Bray, James, Ryman & Thorpe, 1948) that rabbit-liver extracts and slices could bring about hydroxylation of hydroxybenzoic acids and amides as evidenced by the appearance of substances giving colours with ferric chloride. By means of a paper chromatogram of ether extracts of strongly acidified digests both gentisamide and 2:3-dihydroxybenzamide have now been identified as hydroxylation products of salicylamide by liver slices. In the absence of 2:3-dihydroxybenzamide as a reference compound, this amide was identified after hydrolysis to 2:3-dihydroxybenzoic acid which was run alongside an authentic specimen.

SUMMARY

1. A procedure is described for the identification of phenolic compounds by paper chromatography.

2. Examples are given of the use of the method in metabolic studies.

3. All the theoretically possible *o*- and *p*-hydroxylation products have now been identified in rabbit urine after administration of the *o*-, *m*- and *p*-hydroxybenzoic acids and amides.

4. It has been shown that both salicylic acid and *m*-hydroxybenzoic acid are conjugated with glycine in the rabbit.

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Metabolism of Derivatives of Toluene

4. CRESOLS

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The fate of the cresols in the animal body has been studied by various workers. Baumann & Herter (1877–8) observed that commercial cresol gave rise to ethereal sulphate formation in the horse and dog. Williams (1938) found that the percentages of doses of the three isomers excreted conjugated as ethereal sulphate by the rabbit were 22 (ortho and meta), and 16 (para). Neuberg & Kretschmer (1911) demonstrated conjugation of the hydroxyl group in *p*-cresol with glucuronic acid by the isolation, from dog *p*-cresol urine, of a complex of the barium salts of *p*-cresylglucuronide and *p*-cresylsulphuric acid; they were able to separate potassium *p*-cresylsulphate, but not the glucuronide. Jonescu (1906) also showed that the cresols were conjugated with both sulphuric and glucuronic acids in the dog, but could account for only 30–50 % of *o*-, 46·5–50 % of *m*- and 23–27 % of *p*-cresol administered. Preusse (1881) found that in the dog *p*-cresol gave rise to some *p*-hydroxybenzoic acid and *o*-cresol to 2:5-dihydroxytoluene.

The cresols are compounds with one conjugable group and one potential centre for conjugation (Bray, Ryman & Thorpe, 1948), and it was from this point of view that the present investigation was carried out. An attempt has been made to draw up complete balance sheets for the three isomers and to determine whether the same metabolites were formed by the rabbit as by the dog.

METHODS

Diet and dosage

Rabbits (2–3 kg.) were maintained throughout the investigation on the diet of rabbit pellets and water previously described (Bray, Ryman & Thorpe, 1947). The cresols were administered by stomach tube as solutions in $NaHCO_3$. The *o*- and *m*-isomers could be given safely at dose levels of 0·2 g./kg. (dose 500–600 mg.) without further precautions, but the *p*-isomer was more toxic, 200–300 mg. being the maximum safe dose under fasting conditions. If, however, the rabbits were given their daily food 1–2 hr. before administration of the dose, the symptoms of toxicity were much less noticeable and *p*-cresol could be administered at the same level as the other isomers.

Estimation of metabolites in cresol urines

Ether-soluble acid, reducing material and ethereal sulphate were estimated as previously described in the studies of the hydroxybenzoic acids and amides (Bray *et al.* 1947, 1948). The cresylglucuronides are considerably more stable than the glucuronides previously studied: the maximum liberation of reducing material was achieved by heating urine with 10 N-H_2SO_4 (0·5 vol.) in a boiling water bath for 1·5 hr.

Estimation of cresols

The following method, using Folin & Ciocalteu's (1927) phenol reagent was devised for the determination of free and conjugated cresol in urine.

(*a*) *Free cresol.* Urine (20 ml.) as collected (pH 7·8–8·0) was continuously extracted with ether for 6 hr. Water (10 ml.) was then added to the 'boiler' and the ether carefully evaporated. The aqueous residue was then diluted to 25 ml. and portions (1–10 ml., depending on the free cresol content) transferred to boiling tubes, and the volume adjusted to 10 ml. Folin reagent (1 ml.) was then added, followed by 20% (w/v) $NaCO_3$ (2 ml.). The tubes were heated in a boiling water bath for 1 min., cooled in water and the resulting blue solution diluted to 25 ml. and compared with a standard prepared similarly using, instead of the urine extract, 1 ml. of a solution of the corresponding cresol (2 mg./100 ml. made by dilution of a 50 mg./100 ml. aqueous stock solution).

(*b*) *Total cresol.* This estimation was carried out as above using urine hydrolysed by boiling under reflux for 1 hr. with an equal volume of 10 N-H_2SO_4 and then adjusted to pH 7·8–8·0. Control experiments showed that this method gave quantitative recovery of the cresols added to normal rabbit urine, even in the presence of added hydroxybenzoic acid. Attempts to adapt the method to the simultaneous estimation of the hydroxybenzoic acids which might arise by metabolic oxidation of the cresols have so far been unsuccessful. Actually the extent of this oxidation is small and the estimation of the acid produced is rendered more difficult by the fact that the hydroxybenzoic acids give only 36–61% of the intensity of the colour given by equivalent amounts of the corresponding cresols. Further the base line for ether-soluble phenolic acids is comparatively high (about 150 mg./24 hr., expressed as *p*-cresol), so that the actual increases to be expected would represent only a relatively insignificant fraction of the total.

Paper chromatography

This technique was applied, as described by Bray, Thorpe & White (1950), to ether extracts of unhydrolysed and hydrolysed cresol urines.

RESULTS

Quantitative studies

Excretion of normal metabolites. The average daily excretion of normal metabolites by individual rabbits used in this study was as follows: (1) Reducing material expressed as glucuronic acid, 127–226 mg., the day-to-day variation in each experiment usually being less than ± 14% of the weekly mean (average 12%); the average increase in reducing material on hydrolysis was 69%. (2) Ethereal sulphate, 28–47 mg. SO_3, daily variation usually within ± 13% of the weekly mean (average 13%). (3) Free non-acidic phenols (i.e. those extracted at pH 7·8–8·0) expressed as cresols, 6–24 mg., daily variation within ± 25% (± 4 mg.) of the weekly mean (average 20%). (4) Total non-acidic phenols expressed as cresol 40–87 mg., daily variation within ± 15% of the weekly mean (average 11%).

Excretion of free and total cresols, ether glucuronide and ethereal sulphate by the rabbit after administration of cresols. The results obtained are summarized in Table 1. No increase in ester glucuronide excretion was observed. A large number of determinations of ether-soluble acid excretion gave erratic results and are not recorded. As mentioned above, the small percentages of the cresols which appear to be oxidized to the corresponding acids are insignificant in comparison to the normal fluctuations of the ether-soluble acid base line. In early experiments, when the animals were not given their food until after dosage, anorexia almost invariably occurred. This would affect the base-line values and almost cer-

Table 1. *Excretion products of cresols in the rabbit*

	Excretion products			
			Percentage of dose excreted	
Compound administered	Type	No. of experiments	Range	Average
o-Cresol (500 mg.)	Ethereal sulphate	4	13–19	15
	Ether glucuronide	6	55–91	72
	Free cresol	4	0–2	1
	Total cresol	4	75–83	80
m-Cresol (500 mg.)	Ethereal sulphate	5	4–15	10
	Ether glucuronide	6	53–70	60
	Free cresol	6	0–4	1
	Total cresol	6	81–86	84
p-Cresol (250–500 mg.)	Ethereal sulphate	4	12–21	15
	Ether glucuronide	9	32–85	61
	Free cresol	5	0–12	2
	Total cresol	5	52–85	65

tainly reduce the net excretion of all metabolites during the 24 hr. after dosage. This effect was diminished by allowing the animals to satisfy their hunger before dosage, but was not entirely eliminated since even then they did not always consume the whole of their daily ration. It is thus possible that the results obtained are all somewhat low, especially with *p*-cresol, the most toxic isomer. It will be observed from the ranges quoted in Table 1 that *p*-cresol did in fact give more variable results than the other isomers. The excretion of the metabolites under investigation during the second 24 hr. period after dosage was however in general within the normal range.

It can be seen from Table 1 that the extra total non-acidic phenol (i.e. cresol) excretion is approximately equal to the sum of that of ethereal sulphate and ether glucuronide, i.e. that conjugation of the hydroxyl group accounts for the major part of the dose. The discrepancies with the *o*- and *m*-isomers are presumably due mainly to experimental error, since those minor metabolites which have been detected (Table 2) would be included in the total phenol values. The discrepancy with the *p*-isomer must, in part, be due to *p*-hydroxybenzoic acid which is not estimated by the phenol method used, but if conjugated with glucuronic or sulphuric acids would be included in the values for ethereal sulphate and ether glucuronide. Unconjugated *p*-hydroxybenzoic acid is not estimated by any of the chemical methods used: paper chromatography suggested that about 7% of the dose was excreted as free *p*-hydroxybenzoic acid and 3% conjugated.

The total percentages of the cresols for which we accounted are much higher than those found by Jonescu (1906) in the dog. It is possible that his low values were the result of failure to decompose the ether glucuronides during estimation, since we found that values for total phenol little greater than the ethereal sulphate were obtained if we used the method of Volterra (1942), in which the urine is hydrolysed by 2N-H_2SO_4 and the liberated cresol simultaneously removed by distillation.

Excretion of minor metabolites. By means of paper chromatography it was possible to detect and approximately to estimate metabolites which gave phenolic reactions. By comparing the ether extracts of urine before and after hydrolysis, the free and conjugated forms could be distinguished. Experimental details have been given elsewhere (Bray *et al.* 1950). Table 2 lists the possible metabolites and summarizes the results obtained.

Qualitative experiments

The standard lead precipitation method was applied to cresol urines. The *o*- and *m*-isomers yielded syrups which could not be crystallized, but, being sparingly soluble in ether, could be partially purified by continuous extraction with that solvent. The syrups gave a strong naphthoresorcinol reaction, but, on hydrolysis, gave reducing material and cresols identified as cresyl carbanilates. *p*-Cresol urine, however, yielded a crystalline product, m.p. 147° (decomp.), which gave a positive naphthoresorcinol test and, after hydrolysis, a positive Folin & Ciocalteu reaction and reduction with Benedict reagent. (Found: C, 50·6; H, 5·8; glucuronic acid 56·5%. Equiv. (by titration) 299, $[\alpha]_D^{22°} - 71·8°$ (water; *c*, 0·4). $C_{13}H_{18}O_8$ requires: C, 51·7; H, 6·0; glucuronic acid 64·2%. Equiv. 302.) From the products of hydrolysis (boiling under reflux with 2N-HCl for 2 hr.), *p*-cresol was isolated as its benzoyl derivative, m.p. 67–68°. Mixed melting point with authentic specimen (m.p. 70–71°) 68–68°. It was concluded that the compound isolated was the *monohydrate* of p-*cresylglucuronide*.

Isolation of 2:5-*dihydroxytoluene*. This compound was isolated from both *o*- and *m*-cresol urines. The process of isolation was greatly facilitated by examining the fractions at each stage by paper chromatography (Bray *et al.* 1950).

The 24 hr. urine from ten rabbits, each of which had received 0·5 g. of *m*-cresol, was hydrolysed by heating to 90° with 0·1 vol. of conc. HCl for 30 min., and continuously extracted with ether for 24 hr. The ether-soluble material was dissolved in acetone and evaporated under reduced pressure to 20 ml. On chilling, hippuric acid (1 g.) separated and was

Table 2. *Metabolites of cresols in the rabbit detected by paper chromatography*

(++, about 7% of dose; +, about 3% of dose, or less; Tr., trace; −, absent.)

	Metabolites						
	Hydroxybenzoic acid		Position of hydroxyls in dihydroxy compounds	Dihydroxytoluene		Dihydroxybenzoic acid	
Compound administered	Free	Conjugated		Free	Conjugated	Free	Conjugated
o-Cresol	−	−	2:5	−	+	−	−
			2:3	−	−	−	−
m-Cresol	−	−	2:5	−	+	−	−
			2:3	−	−	−	−
			3:4	−	Tr.	−	−
p-Cresol	++	+	3:4	−	Tr.	−	−

removed. The filtrate was treated with charcoal and evaporated to dryness *in vacuo*. The residue was dissolved in the minimum necessary amount of boiling water and the solution made just alkaline with ammonia. The precipitate formed by addition of excess of saturated basic lead acetate was collected, suspended in water (100 ml.) and treated with hydrogen sulphide to remove lead. After addition of excess $Na_2S_2O_4$, the lead-free filtrate was continuously extracted with ether. The ethereal extract was concentrated *in vacuo* to give a syrup which, on treatment with small volumes of water and chilling, gave a crude product, m.p. 105°, consisting mainly of 2:5-dihydroxytoluene. Recrystallization from water gave colourless needles, m.p. 124°, mixed m.p. with authentic 2:5-dihydroxytoluene (m.p. 125°), 125°. Yield 50 mg. (1% of dose). (Found: C, 67·5; H, 6·6. Calc. for $C_7H_8O_2$: C, 67·7; H, 6·5%.) The identity of the isolated metabolite with 2:5-dihydroxytoluene was further confirmed by paper chromatography and by the preparation of the dibenzoyl derivative, m.p. 118–119°, alone or mixed with an authentic specimen. 2:5-Dihydroxytoluene was similarly isolated from *o*-cresol urine. Yield 10 mg. from a total dose of 5 g.

DISCUSSION

The cresols are excreted principally as conjugates through the hydroxyl group, 60–72% as ether glucuronide and 10–15% as ethereal sulphate. Negligible amounts are excreted unconjugated and, as would be expected in compounds possessing a readily conjugable group, little conversion of the potential centre for conjugation or hydroxylation occurs. With regard to the extent to which such processes occur there exists a reciprocal relationship: in *o*- and *m*-cresols, where no conversion of the potential centre could be detected, hydroxylation occurs and in *p*-cresol, of which *p*-hydroxybenzoic acid was shown to be a metabolite, no significant hydroxylation could be detected.

SUMMARY

1. The cresols are excreted by the rabbit mainly as oxygen-conjugates; 60–72% as ether glucuronides and 10–15% as ethereal sulphates.

2. It has been shown by paper chromatography that *o*- and *m*-cresols are hydroxylated to a small extent and that *p*-cresol gives rise to some *p*-hydroxybenzoic acid.

3. *p*-Cresylglucuronide was isolated as a crystalline monohydrate from *p*-cresol urine and 2:5-dihydroxytoluene from *o*- and *m*-cresol urines.

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Further Observations on Changes in β-Glucuronidase Activity in the Mouse

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Increased β-glucuronidase activity in mouse liver or kidney was shown to reflect an increase in the proliferative activity of the tissue (Levvy, Kerr & Campbell, 1948). A similar relationship in uterus has been found to explain changes in the glucuronidase activity of this organ produced by certain sex hormones (Fishman & Fishman, 1944; Fishman, 1947; Kerr, Campbell & Levvy, 1949). Investigation of unexpected changes in glucuronidase activity in mouse tissues led to the discovery of new factors controlling growth in liver and uterus (Kerr *et al.* 1949). Further studies of these factors are described in the present communication. Additional examples of parallelism between the β-glucuronidase activity

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of a tissue and its state of proliferation, with particular reference to the actions of growth inhibitors, are also presented.

EXPERIMENTAL AND RESULTS

Methods

The assay of β-glucuronidase activity in tissue preparations and the measurement of uterine weight were carried out as described by Kerr *et al.* (1949).

The effects of certain steroid hormones on liver and uterine glucuronidase

In a previous communication (Kerr *et al.* 1949) it was shown that oestrone causes a marked rise in glucuronidase activity and in cell division in the livers of ovariectomized mice. This action was also seen in normal and castrated males, but not in intact females. In similar experiments with oestriol and oestradiol (Table 1), no change in liver glucuronidase activity was observed in male or ovariectomized female mice. Both compounds appeared to stimulate cell division in liver, but this effect was seen only with the larger doses studied, and at its greatest was slight compared with that produced by oestrone. Oestriol and oestradiol had the expected stimulant actions on uterine weight and glucuronidase activity (Fishman & Fishman, 1944; Fishman, 1947). It is not impossible that under different experimental conditions these steroids might show an effect on liver enzyme activity.

It has already been noted (Kerr *et al.* 1949) that testosterone antagonizes the effects of oestrone on liver and uterine glucuronidase. As can be seen from Table 1, progesterone (1·8 mg./kg.) antagonized the action of oestrone (1·7 mg./kg.). Given alone, a single injection of progesterone had no effect on the enzyme in liver or in uterus, even when the dose was increased threefold.

No significant changes in kidney glucuronidase activity were observed in the experiments listed in Table 1.

Table 1. *The glucuronidase activity of mouse liver and uterus after injection of certain steroid hormones*

(In this and all subsequent tables values are given as mean ±S.E. followed (in parentheses) by the number of animals in the group. M. = male, F. = female, O.F. = ovariectomized female.

Glucuronidase activity is expressed in units (G.U.). One G.U. liberates 1 μg. phenol from 0·015M-phenylglucuronide in 1 hr. at 38° at pH 5·2 (for liver, kidney and breast tissue) or at pH 4·5 (for uterus).

In the experiments of Table 1 hormones were administered by single subcutaneous injection of solutions in olive oil.)

Treatment	Sex	Days after treatment	Glucuronidase activity (G.U./g. tissue) Liver	Glucuronidase activity (G.U./g. tissue) Uterus	Uterine weight (mg.)
None	O.F.	—	270±18 (18)	166±21 (18)	36±9 (18)
	M.	—	273±14 (12)	—	—
Oestrone (1·7 mg./kg.)	O.F.	1	431±42 (3)	300±54 (3)	26±5 (3)
	O.F.	4	562±33 (9)	524±56 (9)	210±11 (9)
	M.	1	879±98 (9)	—	—
	M.	4	303±12 (6)	—	—
Oestradiol (2 mg./kg.)	O.F.	1	289±13 (3)	231±12 (3)	62±2 (3)
	O.F.	4	301±15 (3)	357±15 (3)	291±4 (3)
Oestradiol (6 mg./kg.)	O.F.	1	277±16 (3)	257±36 (3)	116±7 (3)
	O.F.	4	287±20 (3)	398±23 (3)	273±10 (3)
	M.	1	292±19 (3)	—	—
	M.	4	279±13 (3)	—	—
Oestriol (2·5 mg./kg.)	O.F.	1	281±17 (3)	209±11 (3)	57±6 (3)
	O.F.	4	306±22 (3)	305±15 (3)	257±11 (3)
Oestriol (5 mg./kg.)	O.F.	1	289±22 (3)	451±38 (3)	275±16 (3)
	O.F.	4	279±26 (3)	500±41 (3)	298±21 (3)
	M.	1	281±17 (3)	—	—
	M.	4	295±18 (3)	—	—
Progesterone (1·8 mg./kg.)	O.F.	1	265±29 (3)	156±8 (3)	38±3 (3)
	O.F.	4	284±15 (3)	167±10 (3)	41±4 (3)
Progesterone (5·4 mg./kg.)	O.F.	1	277±19 (3)	172±12 (3)	33±5 (3)
	O.F.	4	272±19 (3)	189±7 (3)	36±4 (3)
Progesterone (1·8 mg./kg.) + oestrone (1·7 mg./kg.)	O.F.	1	266±24 (3)	188±14 (3)	34±4 (3)
	O.F.	4	289±22 (6)	174±13 (6)	32±3 (6)

Changes in uterus during liver regeneration

In absence of administered oestrogen, small but definite increases in uterine weight and glucuronidase activity occur in ovariectomized mice during liver regeneration (Kerr *et al.* 1949). Further experiments (Table 2) show that administration of testosterone or progesterone (by subcutaneous injection in olive oil) prevented the changes in uterus, without influencing the rise in liver glucuronidase activity, after partial hepatectomy. It should be noted that the rise in uterine weight during liver regeneration was never more than about half that caused by an effective dose of oestrogen (Table 1). Vaginal smears were not unlike those obtained with a dose of oestrone, 0·3 mg./kg. (Kerr *et al.* 1949), which caused a similar increase in uterine weight. Vaginal smears taken from ovariectomized mice during liver regeneration were sent to Prof. G. F. Marrian, F.R.S., who gave us the following opinion: 'The smears were not positive on the basis of the Marrian & Parkes (1929) criteria. In many smears there was a lack of leucocytes and considerable mucification. In some there was a lack of leucocytes and some nucleated epithelial cells were present, while in others cornified cells plus leucocytes were observed. The action appeared to be similar to that observed with a dose of oestrogen just below that required to produce a positive smear on the basis of the Marrian & Parkes (1929) criteria.'

Table 2. *The effects of testosterone and of progesterone on uterus in ovariectomized mice after partial hepatectomy*

(Dose divided between two injections, one 6 hr. after partial hepatectomy, the other 3 days later. Measurements made 8 days after partial hepatectomy. G.U. defined in Table 1.)

Treatment	Glucuronidase activity (G.U./g. tissue) Liver	Glucuronidase activity (G.U./g. tissue) Uterus	Uterine weight (mg.)
None	270±18 (18)	166±21 (18)	36±9 (18)
Partial hepatectomy	559±49 (12)	351±30 (12)	111±13 (12)
Partial hepatectomy + testosterone (4·0 mg./kg.)	572±48 (6)	247±27 (6)	43±7 (6)
Testosterone (4·0 mg./kg.)	283±17 (6)	195±15 (6)	32±4 (6)
Partial hepatectomy + progesterone (3·6 mg./kg.)	509±36 (3)	186±16 (3)	38±5 (3)
Progesterone (3·6 mg./kg.)	282±25 (3)	174±21 (3)	35±2 (3)

The glucuronidase activity of proliferating breast tissue

Fishman & Anlyan (1947) found that the glucuronidase activity of human malignant growths was higher than that of most normal tissues. This observation has been confirmed in mice (Karunairatnam, Kerr & Levvy, 1949). Fishman & Anlyan (1947) also noted that the lactating breast in a human subject had a higher glucuronidase activity than normal breast tissue. Table 3 shows the glucuronidase activity of three spontaneous breast tumours in mice, and of healthy breast tissue at various stages of pregnancy and lactation. Histological examination showed the tumours to be adenocarcinomata. In the absence of any data for the kinetics of hydrolysis of phenylglucuronide by breast or tumour glucuronidase, the enzyme assays were done as recommended for liver and kidney (Kerr *et al.* 1949). In each animal, the mammary glands were pooled before homogenizing and taken through the rest of the procedure in the usual way.

Table 3. *Glucuronidase activities of healthy and malignant breast tissue*

(G.U. defined in Table 1.)

Description	Glucuronidase activity (G.U./g. tissue)
Normal adults (virgin)	92±7 (3)
7–14 days pregnant	173±9 (3)
14–21 days pregnant	165±16 (3)
1 day post-partum	151±19 (4)
11 days post-partum	97±4 (3)
Spontaneous adenocarcinomata (individual results)	197, 423, 454

If the figures in Table 3 may be taken as a guide, proliferation of the breast commenced in the mouse in the early stages of pregnancy and had ceased at 11 days post-partum, although the animals were still lactating at that time. At no stage was the glucuronidase activity of healthy breast tissue as high as in the tumours.

The glucuronidase activity of rat liver

It was considered desirable to examine the relationship between the glucuronidase activity and the state of proliferation of a tissue in another animal than the mouse. Table 4 shows figures obtained for the glucuronidase activity of infant, adult, and regenerating rat liver. Assays were done as described for mouse liver (Kerr *et al.* 1949). Liver glucuronidase activity in 4-day-old rats was more than twice the value for normal adults, and enzyme

activity in liver regenerating after partial hepatectomy was even higher. In individual operated animals, the activity of the portion of liver removed was never more than half the figure for the remaining hypertrophied lobe.

Table 4. *Glucuronidase activity of rat liver*

(G.U. defined in Table 1.)

Description	Glucuronidase activity (G.U./g. tissue)
Normal adults	1382±264 (10)
Normal infants (4-day-old)	2977±301 (4)
Adults, 4 days after partial hepatectomy	3177±541 (6)

Effect of growth inhibitors

Colchicine. In view of the inhibitory action of colchicine on growth processes in general (Lits, Kirschbaum & Strong, 1938), a study was made of its action on the glucuronidase activities of various organs in the mouse. The drug was dissolved in 0·9% sodium chloride solution and injected subcutaneously. As can be seen from Table 5, it had no marked effect on the enzyme levels in normal adults, even in a dose of 6 mg./kg., a dose which is usually rapidly fatal.

Given 6 hr. after partial hepatectomy, colchicine (1·5 mg./kg.) prevented hypertrophy of the remaining fragment of liver and the associated rise in glucuronidase activity (Table 5). When the interval between operation and colchicine injection was increased to 24 hr., regeneration took its normal course. Scheifley & Higgins (1940) found colchicine to be effective in partially hepatectomized rats only if given in the initial stages of recovery. Given within a few minutes of oestrone (1·7 mg./kg.), colchicine (1·5 mg./kg.) antagonized the effects of the oestrogen in liver and uterus in ovariectomized mice.

Table 6 shows results obtained in two experiments with infant mice. In each experiment, a litter was

Table 5. *Action of colchicine in combination with measures leading to changes in glucuronidase activity in adult mice*

(G.U. defined in Table 1.)

Treatment	Sex	Days after initial treatment	Glucuronidase activity (G.U./g. tissue) Liver	Kidney	Uterus	Uterine wt. (mg.)
None	M.	—	273±14 (12)	288±22 (12)	—	—
	F.	—	304±29 (12)	306±25 (12)	318±38 (12)	229±27 (12)
	O.F.	—	270±18 (18)	316±22 (18)	166±21 (18)	36±9 (18)
Colchicine (1·5 mg./kg.)	M.	2	273±22 (3)	277±42 (3)	—	—
	F.	2	243±23 (3)	350±45 (3)	213±14 (3)	194±5 (3)
	F.	4	281±18 (3)	379±19 (3)	205±10 (3)	183±6 (3)
	O.F.	2	282±16 (3)	379±31 (3)	187±22 (3)	39±8 (3)
	O.F.	4	269±22 (3)	357±25 (3)	168±24 (3)	29±3 (3)
Colchicine (3 mg./kg.)	M.	1	233±27 (3)	291±22 (3)	—	—
Colchicine (6 mg./kg.)	M.	1	260±15 (2)	252±18 (2)	—	—
Partial hepatectomy	M.	4	780±50 (6)	360±24 (6)	—	—
Partial hepatectomy + colchicine (1·5 mg./kg.) 6 hr. later	M.	4	243±26 (8)	285±17 (6)	—	—
Partial hepatectomy + colchicine (1·5 mg./kg.) 24 hr. later	M.	4	718±68 (3)	280±38 (3)	—	—
Oestrone (1·7 mg./kg.)	O.F.	1	431±42 (3)	313±17 (3)	300±54 (3)	26±5 (3)
	O.F.	4	562±33 (9)	309±20 (9)	524±56 (9)	210±11 (9)
Oestrone (1·7 mg./kg.) + colchicine (1·5 mg./kg.) simultaneously	O.F.	1	307±9 (3)	370±23 (3)	246±32 (3)	32±2 (3)
	O.F.	4	320±20 (6)	362±19 (6)	251±27 (6)	40±8 (6)

Table 6. *Effect of colchicine in young mice*

(Subcutaneous injection of 1 mg. colchicine/kg. in 0·9% NaCl solution every second day. Litter-mate controls injected with pure NaCl solution. G.U. defined in Table 1.)

Description	Age (days) At start	At end	Average body wt. (g.) At start	At end	Increase in wt. (%)	Glucuronidase activity (G.U./g. liver)
Treated	9	18	6·88	6·70	−2·6	347±31 (4)
Controls	9	18	6·73	11·10	64·9	673±49 (4)
Treated	10	15	7·90	8·00	1·3	310±17 (4)
Controls	10	15	8·10	9·21	13·7	1043±62 (4)

removed from the mother and divided into two groups. One group received repeated injections of colchicine and the other was kept as control. Both groups received the same diet. The control animals had gained in weight and their liver glucuronidase activities were normal for their age (Karunairatnam *et al.* 1949) at the end of each experiment. The colchicine-treated mice, on the other hand, did not gain in weight and their liver enzyme levels fell to within the range for normal adults.

Sorbic acid. 'Parasorbic acid' (D-4-hydroxypent-1-ene-1-carboxylic acid lactone) inhibits the growth of connective tissue (Haynes, 1948). It was considered of interest to investigate the possibility that the related compound, sorbic acid (penta-1:3-diene-1-carboxylic acid), which is readily available, might have a depressant action on tissue growth and glucuronidase activity *in vivo*. It has no effect on the enzyme *in vitro* (Karunairatnam & Levvy, 1949). In the following experiments, sorbic acid (Light & Co. Ltd.) was given to mice by subcutaneous injection of a neutralized solution in 0·9% sodium chloride solution.

Single injections of 160 mg. sorbic acid/kg. had no effect on the glucuronidase activities of liver, kidney and uterus in adult mice (male and ovariectomized female), nor was there any striking change in the histological appearance of the organs. The general picture was unchanged when this dose was repeated on several consecutive days (Table 7).

In glucuronidase activity in a severely damaged organ has been noted with other poisons (Levvy *et al.* 1948). The histological findings thus provide some explanation for the action of sorbic acid on the enzyme in liver, but not in kidney.

When the dose of sorbic acid was increased to 360 mg./kg., the mice never survived single injections for more than 2 days, by which time any action on the enzyme was not apparent (Table 7). Histological examination revealed severe necrosis in the liver, slight damage in the kidney, and an arrest of mitosis throughout the body. Repeated daily injections of 240 mg./kg. were also rapidly fatal.

Experiments in which infant mice received 160 mg. sorbic acid/kg. (Table 8) yielded results resembling those obtained with colchicine, in that growth was checked and the liver enzyme activity reduced to the adult value.

Many experiments were carried out in which the action of sorbic acid was studied following partial hepatectomy or injection of oestrone or carbon tetrachloride. Results of representative experiments are shown in Table 9. Sorbic acid did not prevent the rise in liver glucuronidase activity which follows injection of carbon tetrachloride (Levvy *et al.* 1948) nor did it influence the changes produced in liver and uterus by oestrone (Table 1). It did, however, prevent the increase in liver enzyme activity and the associated changes in uterus which follow partial hepatectomy (Table 2). The action of sorbic acid in

Table 7. *Effect of sorbic acid on liver, kidney and uterine glucuronidase in normal mice*

(G.U. defined in Table 1.)

Dosage	Sex	Days after injection	Glucuronidase activity (G.U./g. moist tissue) Liver	Kidney	Uterus	Uterine wt. (mg.)
None	M	—	273±14 (12)	288±22 (12)	—	—
	F.	—	304±29 (12)	306±25 (12)	318±28 (12)	229±27 (12)
	O.F.	—	270±18 (18)	316±22 (18)	166±21 (18)	36± 9 (18)
160 mg./kg. injected daily throughout experiment	O.F.	6	258±26 (3)	382±36 (3)	175±20 (3)	41± 8 (3)
	O.F.	10	263±24 (3)	359±25 (3)	168±16 (3)	35± 7 (3)
240 mg./kg. (single injection)	M.	2	284±37 (3)	361±27 (3)	—	—
	M.	4	27±30 (12)	31±25 (12)	—	—
	M.	5	133±21 (3)	234±19 (3)	—	—
	M.	6	240±29 (3)	345±26 (3)	—	—
	M.	9	287±27 (3)	389±24 (3)	—	—
	M.	11	327±18 (3)	394±31 (3)	—	—
	F.	2	275±21 (6)	360±27 (6)	275±19 (6)	247±17 (6)
	F.	4	17± 9 (12)	34±28 (12)	301±20 (12)	202±14 (12)
360 mg./kg. (single injection)	M.	1	259±26 (3)	370±36 (3)	—	—
	F.	2	245±31 (3)	345±28 (3)	265±19 (3)	209±17 (3)

After a single injection of 240 mg. sorbic acid/kg., a profound depression was seen after 4 days in the glucuronidase activities of liver and kidney, but not of uterus (Table 7). Individual animals sometimes showed zero enzyme activities in liver or kidney. With this dose of sorbic acid, necrosis was seen in the liver, but the kidney was apparently normal. A fall

a dose of 240 mg./kg. in depressing kidney glucuronidase activity, observed after 4 days in normal animals (Table 7), was still seen after partial hepatectomy or injection of carbon tetrachloride, but not after oestrone administration. In the livers of partially hepatectomized mice injected with sorbic acid, cell division was frequently seen to be arrested

Table 8. *Effect of sorbic acid in young mice*

(Subcutaneous injection of 160 mg. sorbic acid/kg. as a neutral solution in 0·9% NaCl solution daily. Litter-mate controls injected with pure NaCl solution. G.U. defined in Table 1.)

Description	Age (days)		Average body wt. (g.)		Increase in wt. (%)	Glucuronidase activity (G.U./g. liver)
	At start	At end	At start	At end		
Treated	10	22	8·10	8·95	10·5	290±22 (4)
Controls	10	22	8·10	13·50	66·6	636±29 (4)
Treated	11	22	6·20	6·66	7·4	283±15 (4)
Controls	11	22	6·15	10·15	65·1	574±32 (4)

Table 9. *Action of sorbic acid in combination with measures leading to changes in glucuronidase activity in adult mice*

(G.U. defined in Table 1.)

Other treatment	Sex	Days after other treatment	Glucuronidase activity (G.U./g. tissue)			Uterine wt. (mg.)
			Liver	Kidney	Uterus	
Single injection of 240 mg. sorbic acid/kg. within 1 hr. of other treatment						
CCl_4 (5·3 g./kg.)	M.	4	576±49 (3)	62±28 (3)	—	—
Partial hepatectomy	M	4	298±32 (6)	78±23 (6)	—	—
Oestrone (1·7 mg./kg.)	M.	4	428±32 (3)	367±35 (3)	—	—
Daily injection of 160 mg. sorbic acid/kg. commencing 3 days before other treatment						
CCl_4 (5·3 g./kg.)	M.	7	858±48 (3)	378±25 (3)	—	—
Partial hepatectomy	O.F.	8	325±36 (3)	347±26 (3)	142±18 (3)	46±9 (3)
	M.	8	307±29 (3)	307±32 (3)	—	—
Oestrone (1·7 mg./kg.)	O.F.	4	468±25 (3)	362±29 (3)	377±25 (3)	197±8 (3)

in the metaphase. Displacement of the chromosomes was occasionally observed in cells in late anaphase.

DISCUSSION

In the variety of experiments described above in which a change was observed in glucuronidase activity, the only other common factor would appear to be a change in the state of proliferation of the tissue studied. The results are thus in accordance with the views put forward concerning this enzyme in previous communications (Levvy *et al.* 1948; Kerr *et al.* 1949; Odell & Burt, 1949). In common with the practice of most other workers in this field, our determinations of glucuronidase activity were made with aqueous extracts of freshly homogenized tissue, after a brief period of incubation to coagulate inactive protein. In recent work, carried out at the Rowett Research Institute, we have found that normal mouse liver contains relatively large amounts of an insoluble glucuronidase precursor. Preliminary results suggest that this precursor disappears from liver when the activity of extracts rises after partial hepatectomy. Incubation at acid pH leads to transformation of the precursor into the soluble enzyme, and this occurs to some extent under the conditions used for the final assay of the enzyme. It is evident that varying the mode of preparation of the enzyme for assay may profoundly alter the type of result obtained during changes in the state of proliferation of a tissue. For example, extracts prepared from tissue homogenates which have first been submitted to prolonged incubation may be expected to contain glucuronidase arising from the precursor as well as the enzyme originally present in an active state.

Colchicine had no effect on the glucuronidase activities of normal adult tissues as measured by our own procedure. It did, however, prevent increases in activity following on measures which normally stimulate cell division. It seems probable from these findings that the action of the drug on the activity of the enzyme was secondary to its effect on mitosis, and that the small number of dividing cells in an adult liver or kidney makes a negligible contribution to the total glucuronidase activity.

Sorbic acid arrested growth in infant mice and liver regeneration in partially hepatectomized mice. In these experiments it kept the liver glucuronidase activity at the normal adult level in the same way as colchicine. The failure of sorbic acid to prevent rises in liver and uterine glucuronidase activity in other experiments may have been due to inadequate dosage. The toxicity of the compound, however, precluded increases in dosage. It is possible that the falls in liver and kidney glucuronidase activity produced in normal mice by 240 mg. sorbic acid/kg. may

have been due, not to the compound itself, but to some metabolite, such as parasorbic acid, since the effects were not seen until 4 days after injection. More information is obviously required regarding the action and fate of sorbic acid in the body before its effects on glucuronidase can be fully understood.

It seems probable that the changes seen in uterus during liver regeneration in ovariectomized mice were due to an extra-ovarian oestrogen since they were antagonized by testosterone and by progesterone. The bearing of this finding on certain experiments which claim to show a change in the metabolism of oestrogens during liver damage and regeneration has already been discussed (Kerr *et al.* 1949). Toxicity for liver may explain the actions of some compounds with feebly oestrogenic properties. It seems quite possible that such compounds could cause urinary excretion of a 'true oestrogen', as defined by Emmens (1943).

The action of oestrone on liver was not shared by oestriol and oestradiol under the conditions of our experiments, but it did resemble an oestrogenic action in that it was antagonized by testosterone (Kerr *et al.* 1949) and by progesterone.

SUMMARY

1. Under similar experimental conditions, oestriol and oestradiol do not show the same action as oestrone (Kerr, Campbell & Levvy, 1949) in increasing liver glucuronidase activity in male and ovariectomized female mice. The action of oestrone on liver is antagonized by progesterone.

2. Testosterone and progesterone antagonize the increases in uterine weight and glucuronidase activity seen in ovariectomized mice during liver regeneration (Kerr *et al.* 1949), without affecting the enhanced enzyme activity in liver itself.

3. The glucuronidase activity of mouse breast tissue rose in the early stages of pregnancy and had returned to normal at 11 days post-partum. At no time was the activity in healthy breast tissue as high as in the mammary tumours studied.

4. Colchicine, itself without action on the glucuronidase activities of normal adult mouse tissues, antagonized the effects of measures which cause an increase in activity. Liver glucuronidase activity in infant mice was reduced to the adult value by colchicine.

5. Sorbic acid inhibits cell division to some extent. This does not, however, explain all the effects of the compound on glucuronidase.

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Urinary Excretion of Pregnanediol* in Human Subjects following the Administration of Progesterone and of Pregnane-3α:20α-diol. 1

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Since the establishment by Venning & Browne in 1937 of the status of pregnane-3α:20α-diol as a metabolic reduction product of progesterone, many figures have been accumulated by different workers on the urinary excretion of pregnanediol in human subjects of both sexes after the injection of the hormone. A critical examination of these figures, however, reveals a somewhat unsatisfactory state of affairs. While there is some measure of agreement that in the majority of human subjects, excluding women during the luteal phase of the menstrual cycle and during pregnancy, the pregnanediol appearing in the urine after the injection of progesterone amounts to approximately 10% of the amount of the hormone administered, wide variations from this rough average have also been reported, and in no one paper are there sufficient figures from any one type of human subject to enable conclusions to be drawn concerning the normal variations which may occur in the conversion of progesterone into urinary pregnanediol. Furthermore, without exception, methods of determining urinary pregnanediol have had of necessity to be employed which are of doubtful accuracy at the low levels of pregnanediol excretion usually encountered in such progesterone metabolism experiments.

These considerations, and the availability of a method for determining urinary pregnanediol, which was believed to be more accurate at pregnanediol excretion levels below about 10 mg./24 hr. than any that had hitherto been described (Sommerville, *et al.* 1948), led the present authors to reinvestigate the conversion of administered progesterone into urinary pregnanediol in human subjects.

In the course of the work described in the present paper the urinary pregnanediol excretion has been studied after the administration of progesterone on 2 successive days to normal men, to normal post-menopausal women with and without pre-treatment with oestrogens, and to hysterectomized women; a comparison has been made in men and in post-menopausal women between the pregnanediol excreted after intramuscularly and orally administered progesterone; and the pregnanediol excretion following the oral administration of pure pregnane-3α:20α-diol to normal men has been determined.

The next paper in this series (Sommerville & Marrian, 1950) describes experiments in which progesterone and pregnane-3α:20α-diol were administered to human subjects over long periods.

* The term 'pregnanediol' is used to denote the material in urine consisting largely, but not necessarily entirely, of pregnane-3α:20α-diol, which is determined by any of the widely used quantitative procedures such as those of Venning (1937, 1938), Astwood & Jones (1941), Guterman (1944, 1945), and Sommerville, Gough & Marrian (1948).

EXPERIMENTAL METHODS

Progesterone dissolved in arachis oil (10 mg./ml.) was administered on 2 successive days, either by intramuscular injection or in gelatin capsules by mouth. Pregnane-3α:20α-diol was administered in solution in arachis oil in capsules by mouth. Subjects for the experiments were selected with great care, particular attention being paid to their willingness to co-operate in the collection of accurate 24 hr. specimens of urine.

Urine specimens (24 hr.) were collected in containers containing 5 ml. of toluene from the subjects in their own homes during a control period of several days prior to the administration of the steroid, during the 2 days of the administration and for the following 5–7 days. Specimens, volumes of which were less than 2500 ml., were made up to that volume with water before withdrawing two 500 ml. samples for the determination in duplicate of the pregnanediol content by the method of Sommerville *et al.* (1948). In the few instances where the 24 hr. volumes were greater than 2500 ml., determinations were carried out on two 500 ml. samples and the appropriate volume correction subsequently applied.

The percentage of administered progesterone, or pregnane-3α:20α-diol excreted as urinary pregnanediol, was calculated after correction of the apparent pregnanediol excretion for the control period 'blank' value.

RESULTS

Pregnanediol excretion by post-menopausal women, hysterectomized women and men injected with progesterone

Determinations of urinary pregnanediol excretion in healthy post-menopausal women injected with progesterone do not seem to have been previously reported, but observations have been reported on other types of women with minimal endogenous progesterone production. Thus Venning & Browne

(1938) recovered 12 % of the administered dose as pregnanediol in the urine of an ovariectomized woman. Cope (1940) found a 6 % recovery in a case of hypoplasia of the uterus and 9 % in a case of anovulatory menstruation, while Jones & TeLinde (1941) report a recovery of 11·5 % in the preovulatory phase of the menstrual cycle.

Venning & Browne (1938) first reported that pregnanediol (as the glucuronide) could not be recovered from the urine after progesterone administration to two hysterectomized women. Later, however, these workers (Venning & Browne, 1940) recovered 9·6 % after administering a larger dose of progesterone to a woman in whom a supravaginal hysterectomy had been performed. Using the same scheme of dosage, 30 mg. on 3 consecutive days, Buxton (1940) recovered 4·1 and 5·4 % from two hysterectomized women, but failed to recover any pregnanediol at all from two similar cases. Following the administration of 40 mg. of progesterone on 3 consecutive days, Jones & TeLinde (1941) reported recoveries of 15·6, 12·9 and 10·9 % of urinary pregnanediol from three hysterectomized women.

The excretion of urinary pregnanediol after the administration of progesterone to men was first reported by Buxton & Westphal (1939) who recovered 17 % from a normal male and very variable amounts from two men with Addison's disease. Hamblen, Cuyler & Hirst (1940) reported a variation in the proportion excreted as pregnanediol of from 0 to 42·5 %, and observed a marked variation in the proportion so excreted when the same subject was investigated on several occasions.

In the present work 60 mg. of progesterone was administered by intramuscular injection on each of 2 consecutive days to six healthy post-menopausal women, three young men and four hysterectomized women. The experiment was repeated on four occasions in one subject (P.). The results are shown in Table 1.

In all these experiments the proportion of the administered progesterone recovered in the urine as pregnanediol ranged from 9·1 to 16·0 %. There was no clear evidence that the percentage recovery was greater in the normal post-menopausal women than in the hysterectomized women and men. It can therefore be concluded that under these particular experimental conditions the post-menopausal uterus plays no significant role in the conversion of administered progesterone into urinary pregnanediol glucuronide. As will be shown in the following paper (Sommerville & Marrian, 1950), however, the uterus of the post-menopausal woman does seem to take a prominent part in the process when subjects are treated daily with progesterone for periods of longer than about 5–8 days.

Table 1. *Urinary pregnanediol after the administration by intramuscular injection of progesterone to post-menopausal women, hysterectomized women and men*

(Progesterone (60 mg.) injected on each of 2 successive days.)

Subject	Age	Type	Percentage of administered progesterone recovered as urinary pregnanediol
E.	65	Normal post-menopausal woman	15·6
H.	70	Normal post-menopausal woman	12·1
McA.	55	Normal post-menopausal woman	13·7
A.	70	Normal post-menopausal woman	15·6
N.	71	Normal post-menopausal woman	16·0
Mc.	60	Normal post-menopausal woman	15·2
M.	68	Hysterectomized post-menopausal woman	13·3
H.	50	Hysterectomized post-menopausal woman	14·1
R.	52	Hysterectomized ovariectomized woman	14·9
L.	42	Hysterectomized ovariectomized woman	9·1
P.	22	Normal man (four experiments)	10·4 10·0 10·8 10·0
J.P.	23	Normal man	14·7
R.	22	Normal man	9·3

It should be noted that, contrary to the findings of Hamblen *et al.* (1940), repetitions of the experiment in the same subject (P.) gave almost identical results.

Pregnanediol excretion by men and post-menopausal women after oral and intramuscular administration of progesterone

Dorfman, Ross & Shipley (1948) reported the excretion of urinary pregnanediol after the oral administration of progesterone to two men, one with diabetes and the other with Addison's disease. Further observations do not seem to have been reported on oral administration of progesterone, nor

has a comparison been made between the proportion of administered progesterone excreted as urinary pregnanediol after oral and intramuscular administration.

In the present work, progesterone (60 mg. on each of 2 successive days) was administered in capsules by mouth to the three men and to two of the post-menopausal women investigated in the previous series. The recovery experiment was repeated on three occasions in one of the male subjects.

In Figs. 1 and 2 the rate of excretion of pregnanediol following the oral administration of progesterone is compared with that following intramuscular injection of the hormone in one male and in one post-menopausal female subject. It will be seen that in these cases, which were typical, oral administration resulted in an earlier and more rapid excretion of pregnanediol than that which followed intramuscular injection. This suggests that the absorption of progesterone in oily solution from the gastro-intestinal tract is more rapid than it is from the site of intramuscular injection.

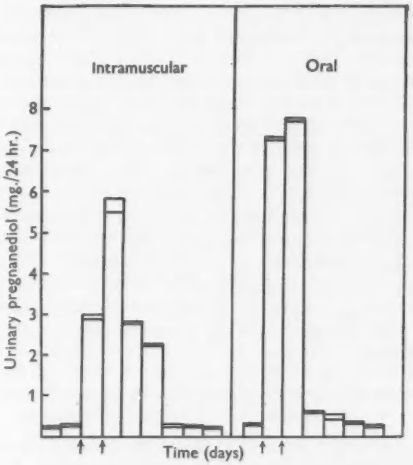

Fig. 1. Urinary pregnanediol excretion after the administration of progesterone to a normal man (subject P.) by intramuscular and oral routes. Arrows indicate administration of 60 mg. progesterone. The values obtained in the duplicate determinations are indicated by the double lines at the tops of the columns.

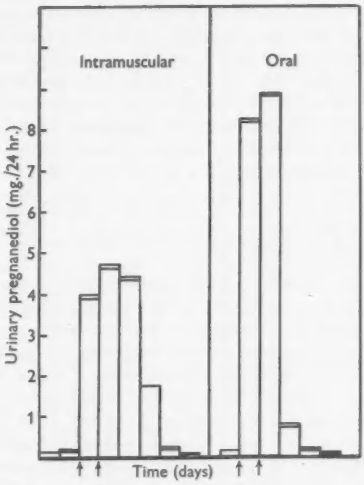

Fig. 2. Urinary pregnanediol excretion after the admistration of progesterone to a post-menopausal woman (subject H.) by intramuscular and oral routes. Arrows indicate administration of 60 mg. progesterone. The values obtained in the duplicate determinations are indicated by the double lines at the top of the columns.

In Table 2 the pregnanediol recoveries following the oral administration of progesterone to the three male and two female subjects are summarized and compared with those obtained from the same subjects after intramuscular injection. Particular attention is drawn to the following two points: (i) the pregnanediol recoveries following oral administration of

Table 2. *Urinary pregnanediol after the administration of progesterone to men and to post-menopausal women by oral and intramuscular routes*

(Progesterone (60 mg.) administered on each of 2 successive days.)

Subject	Age	Type	Percentage of administered progesterone recovered as urinary pregnanediol	
			Oral	Intramuscular
P.	22	Normal man	12·8	10·4
R.	22	Normal man	11·5	9·3
J.P.	23	Normal man (three experiments)	18·6	14·7
			17·4	
			19·6	
N.	71	Post-menopausal woman	18·0	16·0
H.	70	Post-menopausal woman	14·5	12·1

progesterone on three separate occasions to the same subject (J.P.) were virtually the same; (ii) the recoveries following oral administration were in each case slightly higher than the recoveries in the same subjects following intramuscular injection. This latter finding is of some importance in connexion with the use of urinary pregnanediol determinations for clinical diagnostic and prognostic purposes. In view of the well-established fact that the physiological activity of orally administered progesterone, as judged by the rabbit-uterus test, is very small in comparison with its activity by intramuscular injection, this new finding suggests that the amount of pregnanediol in the urine may have little or no quantitative relationship to the amount of endogenous progesterone acting on the target organs.

Pregnanediol excretion after the administration of progesterone to post-menopausal women pre-treated with oestrogen

The evidence in the literature concerning the effect of oestrogen treatment on the urinary excretion of pregnanediol following the administration of progesterone to women is conflicting. Venning & Browne (1940) reported that, in two cases of amenorrhoea with hypoplastic endometria, virtually no pregnanediol could be recovered from the urine. However, after pre-treatment with oestradiol benzoate for several days the same subjects were found to excrete 12 and 18% of the administered hormone as pregnanediol. Venning & Browne (1940) suggested that this effect upon the pregnanediol excretion was due to the 'building up' of the endometrium by the oestrogen. Contrary results were reported shortly afterwards by Cope (1940). In experiments on a similar case he found that pre-treatment with oestradiol benzoate resulted in a slight diminution (6–3%) of the proportion of administered progesterone excreted in the urine as pregnanediol. Evidence of a different character has been advanced by Smith & Smith (1940) to suggest that oestrogen increases the proportion of administered progesterone converted into urinary pregnanediol. They claimed that in toxaemic and diabetic pregnant women progesterone administered alone resulted in a smaller excretion of pregnanediol additional to that arising from endogenous sources than occurred when progesterone was injected together with oestradiol benzoate.

In the present work the problem has been studied in three post-menopausal women with prolapse of the uterus. In two of these (H. and B.) 60 mg. of progesterone was injected intramuscularly on each of 2 successive days, while in the third (K.) the dose was 120 mg./day. The progesterone administration was preceded by the injection of 30 mg. of oestradiol benzoate/day for 6 successive days with subjects H. and B., and for 20 successive days with subject K. The experiment was followed by total hysterectomy in subjects H. and B. and by endometrial biopsy in subject K. Histological section revealed an atrophic endometrium in H., a proliferative endometrium with foci of secretory glands in B. and a proliferative endometrium in K.

The proportions of administered progesterone excreted as pregnanediol were 16·1, 16·2, 15·1% respectively in the three cases. These proportions do not appear to be significantly different from those obtained with post-menopausal women receiving no oestrogen pre-treatment (see Table 1). It may be concluded from these results that under the conditions of these experiments the administration of oestradiol benzoate did not significantly affect the conversion of the administered progesterone into urinary pregnanediol; furthermore, the results suggest that the state of the uterine endometrium had little influence on the conversion. The present authors do not, however, regard the matter as being in any way settled, since it is not improbable that under different experimental conditions oestrogen may influence the conversion of administered progesterone into urinary pregnanediol. It is clear that a final decision on this problem must await the results of further work.

Urinary pregnanediol excretion in men following the oral administration of pregnane-3α:20α-diol

Venning & Browne (1938) were unable to recover pregnanediol glucuronide from the urine of two hysterectomized women after the injection either of progesterone or of pregnane-3α:20α-diol. However, when sodium pregnanediol glucuronidate was injected into the same subjects, 58 and 43% was recovered in the urine. In view of these findings it is surprising that further studies on the urinary excretion of pregnanediol following the administration of pregnane-3α:20α-diol have not been attempted. To the present authors it seemed that such a study might be informative.

Because of the low solubility of pregnane-3α:20α-diol in oil it was not practicable to administer sufficient by injection to give an accurately measurable urinary excretion. Accordingly, administration in oil solution by mouth was adopted. Solutions containing 2·4–3·0 mg./ml. were obtained by dissolving weighed amounts in minimal volumes of boiling ethanol, adding arachis oil, boiling off the excess of ethanol in a water bath and then making up to volume with more arachis oil. Such solutions were administered in measured amounts by mouth in capsules on 2 successive days to three young male subjects whose pregnanediol excretions following the oral administration of progesterone had previously been determined. The results are shown in Table 3. For comparison, the pregnanediol recoveries following the oral administration of progesterone to these subjects are also included in this table.

Table 3. *Urinary excretion of pregnanediol following the oral administration of pregnane-3α:20α-diol to men on 2 successive days*

Subject	Recovery of pregnanediol following the oral administration of progesterone (%)	Pregnane-3α:20α-diol administered orally (mg.)	Recovery of pregnanediol following the administration of pregnane-3α:20α-diol (%)
R.	12·8	2 × 48	12·3
P.	18·6	2 × 56	16·3
J. P.	11·5	2 × 59	13·4

It is seen that the recoveries of pregnanediol in the urine following the oral administration of pregnane-3α:20α-diol are very similar to those obtained in the same subjects following the oral administration of progesterone. So similar are the recoveries in the two sets of experiments that it is tempting to speculate that administered progesterone may be largely reduced to pregnanediol in the body, and that the low recoveries of urinary pregnanediol, following administration of the hormone, may be due to excretion of the reduction product by other routes or further metabolism into products so far unidentified or to a combination of both processes.

SUMMARY

1. Following the intramuscular injection on 2 successive days of progesterone into normal men, normal post-menopausal women and hysterectomized women, 9–16 % of the amount injected is subsequently excreted in the urine as pregnanediol. There is no evidence from these experiments that the post-menopausal uterus plays any significant role in the conversion of progesterone into urinary pregnanediol glucuronide.

2. The urinary excretions of pregnanediol following oral administration of progesterone to normal men and normal post-menopausal women were slightly higher than those observed in the same subjects following intramuscular administration of progesterone.

3. No evidence was obtained to support the view that oestrogen pre-treatment of women with atrophic endometria increases the proportion of administered progesterone excreted in the urine as pregnanediol.

4. Following the oral administration of pregnane-3α:20α-diol to normal men the urinary excretions of pregnanediol were very similar to those observed in the same subjects following oral administration of progesterone.

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Urinary Excretion of Pregnanediol in Human Subjects following the Administration of Progesterone and of Pregnane-3α:20α-diol. 2*

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It has been established by the work of many authors that when progesterone is administered for periods of 1–3 days to human subjects in whom endogenous progesterone production is minimal, the amount of pregnanediol subsequently excreted in the urine accounts for only a small proportion (usually < 20 %) of the administered hormone. In the preceding paper (Sommerville & Marrian, 1950) previous work in this field was comprehensively reviewed and it was shown in an extensive series of experiments on normal men, post-menopausal and hysterectomized women that about 9–16 % of intramuscularly injected progesterone is excreted as urinary pregnanediol, and that a slightly higher proportion of the latter is excreted when progesterone is administered orally.

Venning & Browne (1938, 1940) showed that the administration of progesterone during the luteal phase of the menstrual cycle and during pregnancy, at times when 'the process of conversion of progesterone to pregnanediol was already in action', resulted in the excretion of a much higher proportion of the administered hormone as pregnanediol additional to that arising from endogenous sources. More recently, the greater efficiency of conversion of administered progesterone to urinary pregnanediol by the pregnant woman compared to that of women with minimal endogenous progesterone production has been confirmed by Davis & Fugo (1947), who reported that 30–35 % of progesterone injected during early pregnancy was excreted as 'additional' pregnanediol. These authors came to the important conclusion that the 'activity of the corpus luteum must exert some effect on the metabolism of progesterone so that a much greater percentage can be accounted for as the inert metabolite pregnandiol'.

In view of the possibility that the 'activity of the corpus luteum' responsible, according to Davis & Fugo (1947) for this phenomenon, might be the secretion and physiological action of endogenous progesterone, we felt that some light might be thrown on the problem by studying the urinary pregnanediol excretion of human subjects with minimal endogenous progesterone secretion during periods of prolonged daily administration of progesterone. The only earlier workers who appear to have studied urinary pregnanediol excretion during periods of progesterone administration longer than 3 days are Venning & Browne (1940) and Cope (1940). The former workers administered doses of progesterone of about 10 mg./day for 4–8 days, but the pregnanediol recoveries were irregular and showed no definite daily trend. The findings of Cope, on the other hand, were more definite.

Cope injected a case of secondary amenorrhoea with 10, 5, 5, 5 and 5 mg. of progesterone on 5 consecutive days respectively. Only doubtful traces of pregnanediol were recovered from the urine during the first 5 days, but 2·5 mg. was found on the sixth day. In a second experiment 10 mg. of progesterone was injected daily for 5 consecutive days into a case of anovular menstruation. No pregnanediol at all was detected in the urine during the first 3 days, but a total of 4·5 mg. was found in the urine on the fourth and fifth days. Cope suggested that these findings might be explained on the basis of 'a kind of saturation phenomenon comparable to that which is now well known to occur in the excretion of ascorbic acid taken by mouth', and he furthermore predicted that more prolonged daily treatment with progesterone should result in even higher proportions of the administered hormone appearing in the urine as pregnanediol.

In view of the small doses of progesterone injected by Cope and the relative insensitivity of the method of determining urinary pregnanediol which he employed (Venning, 1937, 1938), it seemed doubtful whether very much quantitative significance should be attached to his results. Nevertheless, his work provided an indication that prolonged administration of progesterone might in some way cause a progressive enhancement of the power of the body to convert progesterone into urinary pregnanediol.

* A preliminary account of certain parts of this work was communicated to the Society for Endocrinology on 21 October 1948 and to the first International Congress of Biochemistry, August 1949 (Sommerville & Marrian, 1949).

EXPERIMENTAL METHODS

The selection of subjects for these experiments, the collection of urine specimens and the determination of the pregnanediol in the latter were, except where stated otherwise, as described in the preceding paper (Sommerville & Marrian, 1950).

Progesterone solutions in arachis oil and in ethyl oleate (10–25 mg./ml.) were administered either by intramuscular injection or in gelatin capsules by mouth. Pregnane-3α:20α-diol was administered in solution in arachis oil in capsules by mouth.

RESULTS

Urinary excretion of 'additional' pregnanediol following the administration of progesterone during pregnancy

In the first instance confirmation was sought of the findings of Venning & Browne (1940) and of Davis & Fugo (1947) that the percentage conversion of administered progesterone into urinary pregnanediol is higher in pregnant women than in human subjects with minimal endogenous progesterone production. This was considered to be necessary, since neither of these groups of workers had provided convincing evidence to show that the endogenous pregnanediol excretion during the control periods in their experiments had been sufficiently constant to justify the calculation of the 'additional' urinary pregnanediol formed from the administered progesterone.

The subject was a woman at the twenty-seventh week of her eighth normal pregnancy who was suffering from mitral stenosis. Since the patient was confined in a busy antenatal hospital ward, reliance could not be placed on the completeness of the collection of 24 hr. urine samples. Accordingly, the creatinine content of the urine collected each day was determined by the method of Folin (1914) and the pregnanediol values corrected to the average creatinine value. In calculating the average creatinine excretion, determinations on the few urine specimens which were obviously incomplete were ignored.

The experiment was carried out over five consecutive 5-day periods as detailed below:

Period 1. Control period for the determination of daily endogenous pregnanediol excretion: total prenanediol excretion = 94·2 mg.

Period 2. Experimental period: 60 mg. progesterone injected on first and second days: total pregnanediol excretion (endogenous + 'additional') = 138·2 mg.

Period 3. Control period as period 1: total pregnanediol excretion = 98·4 mg.

Period 4. Experimental period: 60 mg. progesterone injected on first and second days: total pregnanediol excretion (endogenous + 'additional') = 158·2 mg.

Period 5. Control period as periods 1 and 3: total pregnanediol excretion = 112·4 mg.

The results are shown graphically in Fig. 1. It will be seen that the daily endogenous pregnanediol excretions during each of the three control periods were remarkably constant. It was therefore felt to be justifiable to assume for the purpose of calculating the 'additional' pregnanediol excreted during the two experimental periods that the endogenous pregnanediol excretion during period 2 would be fairly

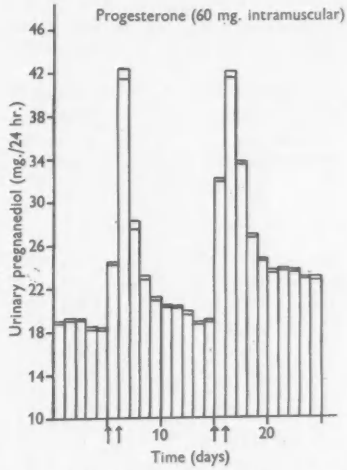

Fig. 1. Effect of progesterone on the excretion of pregnanediol during pregnancy.

represented by the average of those during periods 1 and 3, while that during period 4 would be the average of those during periods 3 and 5. Thus the 'additional' pregnanediol excreted during period 2 is equal to

$$138{\cdot}2 - \left(\frac{94{\cdot}2 + 98{\cdot}4}{2}\right)$$

$= 41{\cdot}9$ mg.

$= 34{\cdot}9\,\%$ of the administered progresterone,

and the 'additional' pregnanediol excreted during period 4 is equal to

$$158{\cdot}2 - \left(\frac{98{\cdot}4 + 112{\cdot}4}{2}\right)$$

$= 52{\cdot}8$ mg.

$= 44{\cdot}0\,\%$ of the administered progesterone.

These results clearly confirm the earlier findings of Venning & Browne (1940) and of Davis & Fugo (1947). Provided, therefore, that the administration of progesterone does not stimulate the secretion of endogenous progesterone, it can be concluded that a much higher percentage of administered progesterone is converted into urinary pregnanediol by the pregnant woman than by human subjects in whom the endogenous secretion of progesterone is minimal.

Urinary pregnanediol excretion during continued daily administration of progesterone to normal post-menopausal women

Preliminary experiments on pregnanediol excretion during continued daily administration of progesterone were carried out upon two post-menopausal women. One (F.) received 60 mg. progesterone per day intramuscularly for 10 days, while the other (N.) received the same daily dose administered orally for 15 days. The results are shown

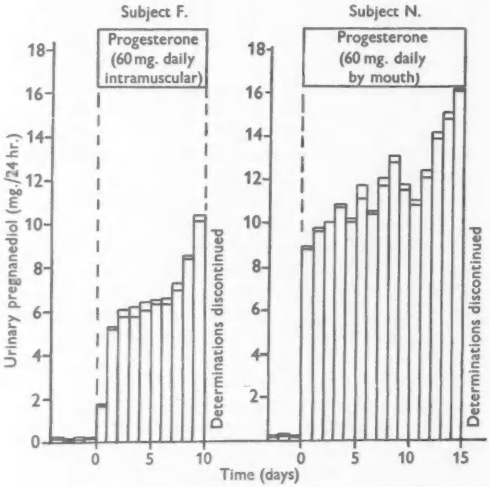

Fig. 2. Pregnanediol excretion during prolonged administration of progesterone to normal post-menopausal women.

graphically in Fig. 2. It will be seen that in subject F. the daily pregnanediol excretion was maintained at an almost constant 'plateau' level, corresponding to about 10 % of the daily dose of progesterone, between the third and eighth days. The pregnanediol excretion then rose sharply, and by the tenth day, when the experiment was discontinued, it had reached a value corresponding to 17 % of the daily dose of progesterone. It will also be seen that a similar phenomenon occurred in subject N. who received the progesterone orally. In this experiment the pregnanediol excretion was somewhat irregular, but it will be clear that after reaching a rather ill-defined plateau level corresponding to about 17 % of the daily dose of progesterone (second to sixth day), the pregnanediol excretion rose to 25 % of the daily dose of progesterone by the fifteenth day. These preliminary results indicated that in post-menopausal women treated with progesterone, either by injection or orally, the power to convert the administered hormone into urinary pregnanediol becomes considerably enhanced after about 6–8 days of the treatment.

In order to confirm this interesting finding and to see whether a second 'plateau' of pregnanediol excretion would ultimately be obtained four further experiments of longer duration were carried out upon three more post-menopausal women. The details of these experiments and the results obtained are shown in Table 1 and Fig. 3.

It will be seen from Fig. 3 that in every experiment the daily pregnanediol excretion rose considerably after reaching a temporary initial 'plateau' level and ultimately attained a second 'plateau'. The results of the two preliminary experiments were thus amply confirmed and extended. This rise in pregnanediol excretion from the initial 'plateau' level will hereafter be referred to as a 'priming' effect.

It will be seen from Table 1 that the 'priming' effect, expressed as the percentage increase of the final 'plateau' level above the initial 'plateau' level, was greatest in the experiment in which the progesterone was administered intramuscularly. It may also be pointed out that in the three experiments in which oral administration was employed, a dose of 60 mg./day produced a greater 'priming' than was obtained with 40 mg./day. As will be seen later, these observations may be of some significance.

Urinary pregnanediol excretion during continued daily administration of progesterone to normal men

The fact that marked 'priming' was observed in post-menopausal women receiving progesterone orally suggested at first that the effect was probably not associated in any way with the physiological action upon the uterus, since it is generally recognized that orally administered progesterone shows only a small fraction of the physiological activity, as judged by the rabbit uterus test, of that shown by the injected hormone. Furthermore, previously reported experiments (Sommerville & Marrian, 1950) in which progesterone was administered by injection on 2 successive days to post-menopausal and to hysterectomized women had suggested that the post-menopausal uterus might have no significant role in the conversion of administered progesterone into urinary pregnanediol. It was confidently expected, therefore, that a 'priming' effect, quantitatively similar to that observed in post-menopausal women, would be obtained in men.

Experiments were therefore carried out on three normal men as follows:

Subject M., aged 30; 40 mg. progesterone per day orally for 18 days.

Subject R., aged 62; 60 mg. progesterone per day orally for 18 days.

Subject P., aged 22; 60 mg. progesterone per day orally for 18 days.

Table 1. *Urinary pregnanediol in post-menopausal women during prolonged treatment with progesterone*

Subject	Daily dose of progesterone and route of administration (mg.)	Duration of progesterone treatment (days)	Pregnanediol excretion: Initial plateau (as % of daily dose of progesterone)	Pregnanediol excretion: Final plateau (as % of daily dose of progesterone)	Pregnanediol excretion: Increase (final plateau—initial plateau, as % of initial plateau)
H.	50 (intramuscular)	22	10·0	22·5	125
H.	60 (oral)	21	14·0	24·0	71
D.	40 (oral)	22	25·5	36·0	41
G.	40 (oral)	27	15·0	20·0	33

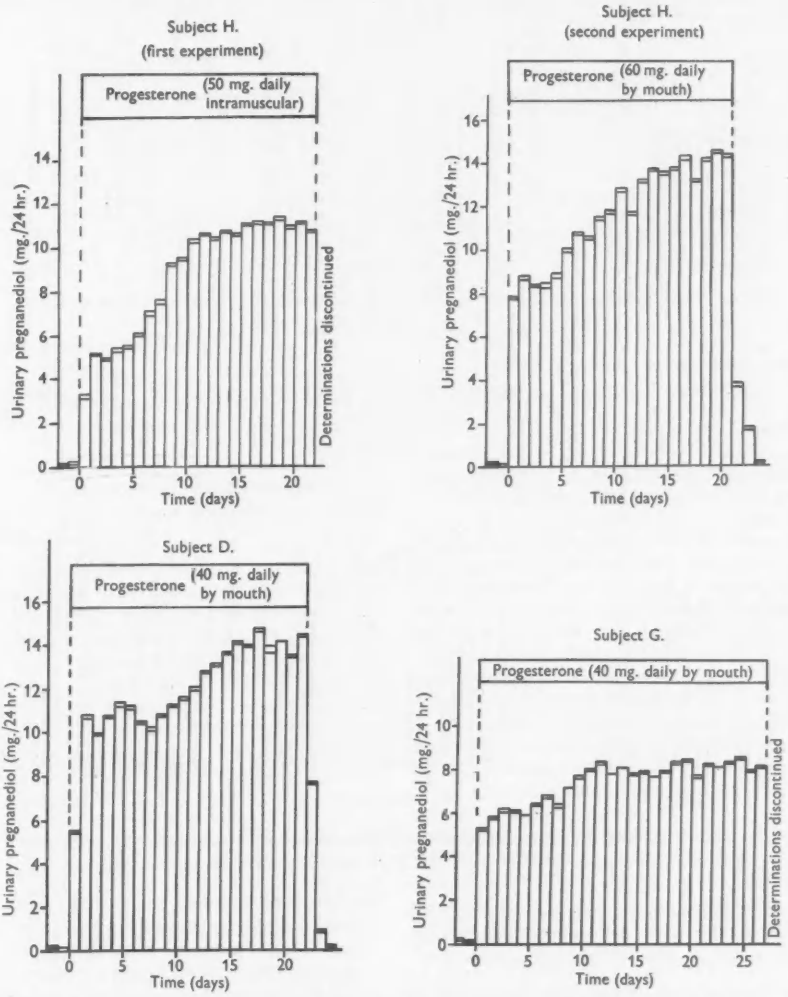

Fig. 3. Pregnanediol excretion during prolonged administration of progesterone to normal post-menopausal women.

The results, which are shown graphically in Fig. 4, clearly show that no 'priming' whatsoever occurred in these three men.

menopausal uterus may have no role in the conversion of administered progesterone to urinary pregnanediol in experiments of only a few days' duration,

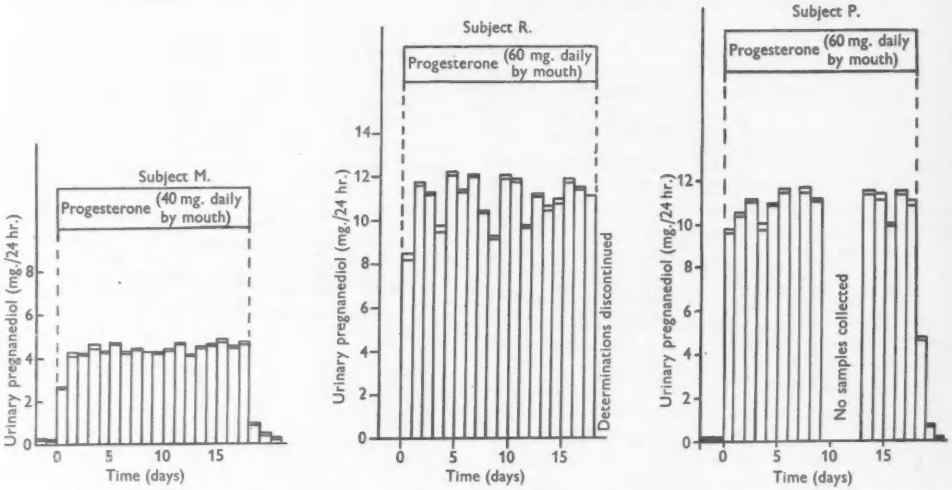

Fig. 4. Pregnanediol excretion during prolonged administration of progesterone to normal men.

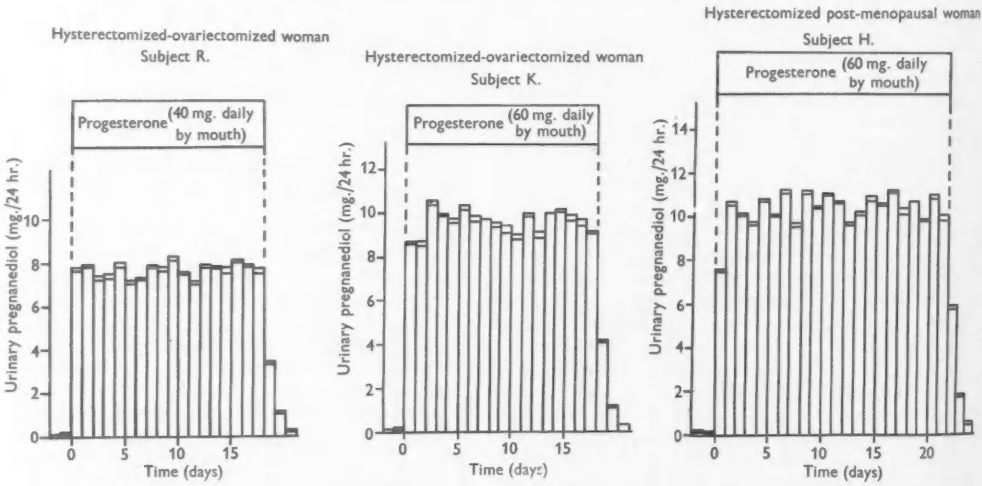

Fig. 5. Pregnanediol excretion during prolonged administration of progesterone to hysterectomized-ovariectomized and hysterectomized post-menopausal women.

Urinary pregnanediol excretion during continued daily administration of progesterone to hysterectomized (ovariectomized or post-menopausal) women

The unexpected lack of any 'priming' effect in men suggested the possibility that although the post-

it might nevertheless be responsible for the 'priming' observed in post-menopausal women after administration of progesterone for longer periods.

Accordingly, experiments as detailed below were carried out upon three women who had been hysterectomized not less than 2 months previously.

Subject R., aged 42; hysterectomized, ovariectomized; 40 mg. progesterone per day orally for 18 days.

Subject K., aged 45; hysterectomized, ovariectomized; 60 mg. progesterone per day orally for 18 days.

Subject H., aged 50; hysterectomized, post-menopausal; 60 mg. progesterone per day orally for 22 days.

The results shown in Fig. 5 indicate quite definitely the complete lack of any 'priming' in these three cases. There can be little doubt therefore that the uterus is necessary for the progesterone 'priming' effect observed in post-menopausal women.

The results, shown in Fig. 6, indicate clearly that no 'priming' occurred as the result of continued daily administration of pregnane-3α:20α-diol.

DISCUSSION

It is clear from the previous work of others and of the present authors that the uterus is not essential for the conversion of administered progesterone into urinary pregnanediol; nor does it seem that the normal post-menopausal uterus plays any significant role in the conversion process when progesterone is administered over periods of only a few days (cf. Sommerville & Marrian, 1950). In the present work, however, it has been clearly demonstrated that the enhanced power of the normal post-menopausal woman to excrete urinary pregnanediol after prolonged daily administration of progesterone depends upon the uterus. Accordingly, the conclusion can hardly be avoided that the post-menopausal uterus after prolonged exposure to the action of progesterone or of one of its metabolic products is able to effect the conversion of progesterone into urinary pregnanediol, or at least some part of that conversion.

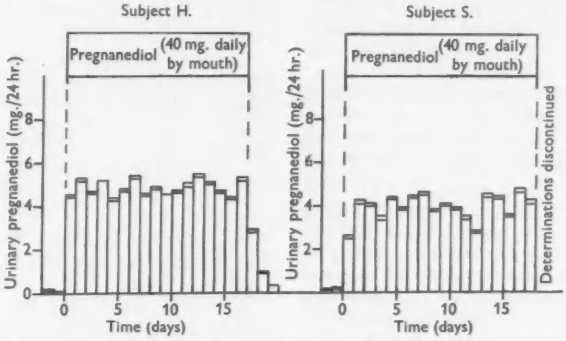

Fig. 6. Pregnanediol excretion during prolonged administration of pregnane-3α:20α-diol to normal post-menopausal women.

Urinary pregnanediol excretion during continued daily administration of pregnane-3α:20α-diol to normal post-menopausal women

In view of the possibility, which is discussed later in this paper, that the 'priming' phenomenon in post-menopausal women may be associated with the action of progesterone in causing structural changes in the uterus, it seemed of importance to test for 'priming' activity related steroids which are known or suspected to be progesterone metabolites, but which lack the physiological activity of the hormone. Unfortunately, owing to the lack of sufficient quantities of other possible progesterone metabolites, pregnane-3α:20α-diol is the only one of these steroids which could be tested.

Experiments, as detailed below, were carried out on two normal post-menopausal women, one of whom (H.) had previously shown definite 'priming' with progesterone.

Subject H., 40 mg. pregnane-3α:20α-diol per day orally for 17 days.

Subject S., 40 mg. pregnane-3α:20α-diol per day orally for 18 days.

At the present time there is no definite evidence to show whether this 'priming' effect of progesterone is, or is not, associated with its physiological action in inducing structural changes in the uterine endometrium and myometrium. The observation that the 'priming' was greater with intramuscularly than with orally administered progesterone, and the observation that in the three oral administration experiments the higher dose gave the greatest 'priming', suggest that perhaps this may be the case. However, the figures at present available are obviously too few to constitute clear-cut evidence in favour of this view. Furthermore, it may be

doubted whether 40 mg. of progesterone per day administered orally without oestrogen would induce any structural changes in the uterine endometrium or myometrium.

The observation that the oral administration of pregnane-3α:20α-diol gave no 'priming' in post-menopausal women is compatible with the theory that the effect may be due to progesterone *per se*. The possibility must be borne in mind, however, that 'priming' might result from other progesterone metabolites which have not yet been tested.

It is possible, but by no means certain, that the increased excretion of pregnanediol observed by Cope (1940) after 5–6 days administration of progesterone may have been due to the same 'priming' phenomenon which is reported here. The possibility that the 'priming' effect might be due to a 'saturation phenomenon', such as was suggested by Cope (1940), must therefore be briefly considered.

The fact that in men and in hysterectomized women a steady 'plateau' of pregnanediol excretion is established within 2–3 days after the commencement of progesterone administration and is maintained for periods up to 22 days indicates that there can certainly be no 'saturation phenomenon' in the absence of the uterus. It is, furthermore, unlikely for two reasons that the 'priming' in normal post-menopausal women could be due to a saturation of the uterine tissue with progesterone and/or its metabolites. First, if such a saturation were to occur, then in experiments of short duration the pregnanediol excretion should be relatively lower in normal post-menopausal women than in hysterectomized women. As has been previously shown, however, the presence or absence of the uterus seems to have little effect on the excretion of pregnanediol in experiments in which progesterone is administered for 2 days only (Sommerville & Marrian, 1950). Secondly, if the 'priming' effect were due to saturation of the uterine tissue with progesterone and/or its metabolites, one would expect that the clearance of pregnanediol, as judged by its rate of disappearance from the urine after stopping the administration of progesterone, would be delayed in the experiments of long duration in comparison with those of short duration. No such delay in pregnanediol clearance in experiments of long duration was in fact observed (see Fig. 3, Subject H., second experiment).

It is not unlikely that the relatively high percentage conversion of administered progesterone into 'additional' urinary pregnanediol which occurs during pregnancy and also possibly during the luteal phase of the menstrual cycle may be due to a 'priming' of the uterus with endogenous progesterone. At the present time, however, it would be premature to assume that the two phenomena are necessarily due to the same cause.

SUMMARY

1. Previous findings by Venning & Browne (1940) and Davis & Fugo (1947) that the pregnant woman can convert a higher proportion of administered progesterone into urinary pregnanediol than can human subjects in whom endogenous progesterone production is minimal have been confirmed.

2. It has been shown that when progesterone is administered daily (intramuscularly or orally) to normal post-menopausal women for periods up to 27 days, the daily urinary pregnanediol excretion reaches on the second or third day a preliminary plateau level which is maintained until the fifth to eighth day. The daily pregnanediol excretion then rises and at about the twelfth to sixteenth day reaches a second plateau which is then maintained. This phenomenon has been termed a 'progesterone "priming" effect'.

3. No 'priming' effect was observed when progesterone was administered daily to normal men, hysterectomized-ovariectomized and hysterectomized post-menopausal women. It has been concluded, therefore, that the uterus is necessary for the manifestation of progesterone 'priming'.

4. No 'priming' effect was observed when pregnane-3α:20α-diol was administered daily (orally) to normal post-menopausal women.

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Some Properties of Acridane and 2-Chloro-7-methoxyacridane: their Possible Relationship to Excretion Products of Mepacrine*

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The separation of basic excretion products of mepacrine (2-chloro-5-(4′-*N*:*N*-diethylamino-1-methylbutylamino)-7-methoxyacridine dihydrochloride) from human urine has been reported previously (King, Gilchrist & Tárnoky, 1946). A number of degradation products appeared, on spectrographic evidence, to be diphenylamine derivatives, but the possibility of their being acridanes could not be excluded. The latter are electronic analogues of diphenylamines, resonance in the centre nucleus having been destroyed. They are known to be similar in their molecular refractions (Auwers & Kraul, 1925*a*, *b*) and may be expected to have similar absorption spectra. Moreover, the change from mepacrine to an acridane derivative is smaller than would be the rupture of the pyridine ring in acridine.

On the other hand, the relatively low stability of acridanes made their presence in the urine or the urine extracts unlikely. Furthermore, our method of separation involved an extraction with hydrochloric acid (0·5N) in which acridanes are generally considered to be insoluble. On neither of these two points, however, were quantitative data available.

The aim of this work was to obtain such data regarding the stability of two representative acridanes and their solubility in 0·5N-hydrochloric acid. Acridane was chosen as the most readily accessible compound of its class, and 2-chloro-7-methoxyacridane as one closely related to mepacrine. The ultraviolet absorption spectra of these two compounds were also determined. They are reproduced here for comparison with the spectra of diphenylamines and of mepacrine degradation products published in the previous paper.

EXPERIMENTAL

Acridane. 5-Chloroacridine (5 g.) in alkaline aqueous-ethanolic-benzene solution was hydrogenated in the presence of a large excess of Raney Ni (3·5 g.), as described by Albert & Willis (1946). H_2 uptake: 1180 ml. at 24° (theory for 4H: 1100 ml. at 15°). Air was admitted, ethanol (100 ml.) added, and the mixture heated on the steam bath in an atmosphere of N_2, and quickly filtered.

On evaporation, the filtrate yielded a crystalline residue which was heated with 2N-HCl on the steam bath for 10 min. The acid-insoluble solid, separated by filtration, was twice recrystallized from hot acetone-water. Yield: 3·5 g., m.p. 168–170° (corr.). Albert & Willis (1946) give m.p. 169–170°.

The identity of this substance was further confirmed by comparison with a specimen of acridane obtained by Kane (1950) by controlled-potential electrolytic reduction of acridine. This specimen melted at 168–169°; admixture with the acridane, prepared as described above, gave m.p. 167–169° (both m.p. uncorr.).

2-Chloro-7-methoxyacridane. 2:5-Dichloro-7-methoxyacridine (6·5 g.) was hydrogenated in the same way as above, but more benzene (50 ml.) and ethanol (200 ml.) were used. The residue from the filtered hydrogenation product was powdered, heated in 200 ml. 2N-HCl at 80° for 10 min. and separated by filtration. It was heated in acetone solution with animal charcoal, filtered and recrystallized from aqueous acetone. One more acid extraction followed by one recrystallization from aqueous acetone and two from benzene-light petroleum yielded the pure 2-chloro-7-methoxyacridane as a pale yellow microcrystalline powder, m.p. 185–186° (corr.) in a sealed tube. (Found: N, 5·9; Cl, 14·9. Calc. for $C_{14}H_{12}ONCl$: N, 5·7; Cl, 14·5%.)

This compound has been obtained by a different method by Hall & Turner (1945) who give m.p. 187–188°.

Stability of the acridane in aqueous-ethanolic solutions at room temperature

Approximately 0·01M-solutions of each acridane were prepared in 90% ethanol–10% water solution, and in 90% ethanolic–10% aqueous NaOH and HCl of 0·01N- and 0·1N-final concentrations respectively. These solutions were allowed to stand at room temperature in dark, loosely stoppered bottles, for a period between November and January. The amount of unchanged acridane was estimated, by the method described below, in portions withdrawn periodically from the solutions.

Estimation of acridanes

A portion of the solution (20 ml.) was diluted with ethanol (10 ml.). Na acetate (anhydrous 0·3 g.) and 2N-HCl (0·3 ml. to the neutral, 0·4 ml. to the alkaline and 0·1 ml. to the acid solutions) were added to give a pH 5·6–5·8; this was followed by 0·1N-I_2 (10 ml.). The solution was allowed to stand in a glass-stoppered flask at room temperature for 4 days, by which time oxidation of the acridane by iodine was complete. The excess I_2 was determined by means of 0·05N-$Na_2S_2O_3$ solution, using a starch phenol red indicator.

Solubility of acridane in aqueous 0·5N-hydrochloric acid

(i) '*Hot.*' An excess of acridane was added to 0·5N-HCl at 80° and the mixture was shaken mechanically and allowed to cool. Room temperature (21°) was reached in

* A preliminary report of this work was presented at a meeting of the Biochemical Society held at the Postgraduate Medical School, London, W. 12, on 21 January 1950 (Tárnoky, 1950).

1 hr. A weighed amount (about 3 g.) of the saturated solution, obtained by filtering into a 5 ml. weight pipette, was washed into a flask by means of ethanol; Na acetate (anhydrous, 5 g.) was added, followed by 0·004 N-I_2 (30 ml.). The solution was allowed to stand at room temperature for 10 days, and the excess I was determined by titrating with 0·005 N-$Na_2S_2O_3$. Solubility of acridane at 21° in 0·5 N-HCl: 42 mg./1000 g. solution.

(ii) '*Cold.*' Ice-cold 0·5 N-HCl was shaken mechanically with acridane until room temperature was reached. The same procedure as in (i) gave the solubility at 21° as 55 mg./1000 g. solution.

Solubility of 2-chloro-7-methoxyacridane in aqueous 0·5 N-hydrochloric acid

Determinations (i) and (ii) were attempted as above. All the added I_2 (30 ml. of 0·004 N-solution) was used up in both experiments.

Spectrophotometry

Absorption spectra in the ultraviolet and visible range were recorded by Mr W. R. Weedon using a Beckman spectrophotometer. The acridanes were examined in ethanolic solution and also in 50% ethanolic–50% aqueous solutions containing 0·5 N-HCl or 0·5 N-NaOH (final concentrations), and at pH 7·6 using Palitzsch boric acid borax buffer (Britton, 1942). The approximate ages of the solutions at the commencement of the spectrum determinations were as follows: acridane at pH 7·6, 3 hr.; acridane in NaOH, 1 hr.; acridane in HCl, 20 min.; all solutions of 2-chloro-7-methoxyacridane, 15 min.

RESULTS

The stability of acridane solutions

The rates of decomposition of six solutions are shown in Table 1. The iodine titres have been recalculated to refer to exactly 0·01 M-solutions of acridanes. These results are expressed as percentages of unchanged acridane or 2-chloro-7-methoxyacridane, '100%' referring to an unchanged acridane solution of 0·01 M-strength.

Absorption spectra

Acridane (Fig. 1). The spectrum of this compound in ethanol solution shows a single wide absorption band with $\lambda_{max.}$ at 2900 A. and $\epsilon \times 10^{-3} = 13{\cdot}68$ (or $\log \epsilon = 4{\cdot}136$). These values agree well with the data obtained by Blout & Corley (1947) who record a single peak at 2890 A. ($\log \epsilon = 4{\cdot}14$). These workers note the agreement of this curve with that of diphenylamine (Castille, 1926).

The solutions in hydrochloric acid, sodium hydroxide and at pH 7·6 all show the same absorption maximum at 2900 A. None of these curves, however, are pure acridane spectra. An inflexion at 2500 A. of the ethanolic solution becomes well marked in sodium hydroxide, while the hydrochloric acid solution shows the sharp absorption band of the acridine spectrum with $\lambda_{max.}$ at 2550 A. (King *et al.* 1946). The long wave band of acridine ($\lambda_{max.}$ 3550 A.)

Table 1. *Stability of 0·01 M-acridane and 2-chloro-7-methoxyacridane solutions at room temperature*

Age of solution	Percentage of original acridane content
Solution 1. Acridane in 10% water–90% ethanol	
0·5 hr.	104
5	100
23	94
4 days	86
8	70
12	62
20	49
29	30
36	20
50	6
64	7
Solution 2. Acridane in 10% aqueous–90% ethanolic 0·01 N-NaOH	
0·5 hr.	102
3·5	101
2 days	92
5	87
8	82
14	78
23	66
30	62
44	54
58	50
Solution 3. Acridane in 10% aqueous–90% ethanolic 0·1 N-HCl	
3 hr.	29
24	9
7 days	11
16	0
23	0
Solution 4. 2-Chloro-7-methoxyacridane in 10% water–90% ethanol	
0·5 hr.	100
3	93
24	31
3 days	0
16	0
Solution 5. 2-Chloro-7-methoxyacridane in 10% aqueous–90% ethanolic 0·01 N-NaOH	
0·5 hr.	85
6	79
28	64
52	53
15 days	22
20	18
26	18
33	18
Solution 6. 2-Chloro-7-methoxyacridane in 10% aqueous–90% ethanolic 0·1 N-HCl	
0 hr.	32
24	10
14 days	5
19	3
25	3
32	5

is also well developed, and the solution fluoresces whitish blue. Generally, acridane is seen to decompose faster in acid than in alkaline solution, and

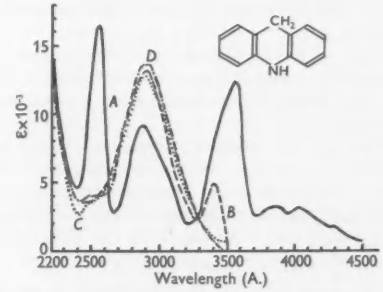

Fig. 1. Spectral absorption curves of acridane solutions. Curve *A* was determined starting measurements at 2200 A. and proceeding steadily to 4500 A. The $\lambda_{max.}$ at 2550 A. represents approx. 12% conversion to acridine in a solution about 30 min. old; the $\lambda_{max.}$ at 3550 A. corresponds to the presence of approx. 65% acridine about 30 min. later. *A*, ——, 0·5 N-HCl (50% ethanol); *B*, – – – –, pH 7·6 (50% ethanol); *C*, ..., 0·5 N-NaOH (50% ethanol); *D*, –·–·–·–, ethanol.

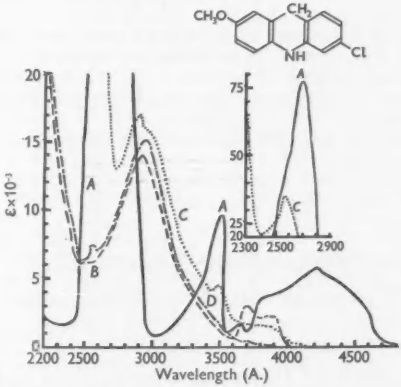

Fig. 2. Spectral absorption curves of 2-chloro-7-methoxyacridane solutions. Curves *A* (determined starting from 4800 A. and proceeding to 2200 A.) and *C* (determined from 2300 A. towards 4200 A.) show more marked changes than the corresponding curves in Fig. 1. *A*, ——, 0·5 N-HCl (50% ethanol); *B*, – – – –, pH 7·6 (50% ethanol); *C*, ..., 0·5 N-NaOH (50% ethanol); *D*, –·–·–·–, ethanol.

least in ethanol. The 'growth' of the peak at 2550 A. would suggest that this band is not part of the acridane spectrum, and that even the ethanol solution, in common with that of Blout & Corley (1947), contains a trace of acridine. This peak does not appear in the spectrum of diphenylamine.

2-Chloro-7-methoxyacridane (Fig. 2). A wide absorption band with $\lambda_{max.}$ at 2920 A. is present in the ethanol, sodium hydroxide and pH 7·6 spectra ($\epsilon \times 10^{-3}$ in ethanol = 15·1 at 2920 A.; $\epsilon \times 10^{-3}$ in sodium hydroxide = 16·7 at 2920 A.) but is not observed in the acid solution within 15 min. In addition to the two bands characteristic of acridine, this solution shows a weak absorption band at 3150 A. and a larger, diffuse band in the 3700–4600 A. region, with $\lambda_{max.}$ at 4200 A. The single absorption peak of 2-chloro-7-methoxyacridane itself is very similar to that of the parent acridane but this spectrum is considerably blurred by the superimposed acridine bands. The chloromethoxy compound changes visibly, more so than acridane. The acid solution is yellow from the start. At 4000 A. the 15 min. old sodium hydroxide solution has $\epsilon \times 10^{-3} = 0{\cdot}56$; 30 min. later $\epsilon \times 10^{-3} = 1{\cdot}25$. As in the case of acridane, the ethanolic solution is the most stable, the one in acid the least stable. In both cases, the spectra in hydrochloric acid are included as giving an indication of the instability of these compounds rather than from the point of view of the spectroscopy of pure acridanes.

DISCUSSION

The outstanding characteristic of these two compounds is their instability. This property is demonstrated by the behaviour of their ethanolic solutions at room temperature; it also interferes with the measurement of their acid solubilities and absorption spectra. Under the conditions selected, acridane is more stable than its 2-chloro-7-methoxy-analogue. Both are more stable in alkaline than in acid solution. This is shown by their absorption spectra, which are as much a measure of the instability of these compounds as of their optical properties. Thus simple acridanes are too unstable to survive unchanged in the voided urine or throughout the extraction although stabilized forms (cf. compounds substituted in the 5 and 10 position) might survive. The change undergone by acridane is clearly an oxidation to acridine. In the case of the chloromethoxy compound the spectrum of the corresponding acridine is not available for comparison, and the acid spectrum may indicate some further or additional reaction, but even here the primary change is probably to form 2-chloro-7-methoxy-acridine. While, therefore, the diphenylamine derivatives referred to in the previous paper may have been formed by way of acridanes, it is most unlikely that this latter stage of the reaction took place during the extraction procedure.

On the basis of spectrographic evidence the acridanes are not similar to any of the mepacrine fractions or to the particular diphenylamine derivatives previously examined. A stabilized form (for in-

stance of 5:10-dihydromepacrine) may of course have a substantially different spectrum. It is possible that such a compound would absorb at higher wavelengths than both mepacrine and its degradation products do. The possible formation of a diphenylamine derivative retaining the mepacrine side chain was suggested in the first paper.

The results of the solubility experiments must also be considered in conjunction with the low stability of these compounds. A solution containing about 50 mg./l., although much too dilute for preparative work, would suffice for spectroscopy and, given stability, the correct spectrum in the ultraviolet region could be obtained. The result with acridane, however, suggests that the slowly dissolving compound is simultaneously oxidized into the stable and soluble acridine, with some unchanged acridane present at the time of the solubility determination, possibly as the hydrochloride of the molecular compound between acridine and acridane suggested by Blout & Corley (1947). The unexplained result obtained with the chloromethoxy compound is presumably connected with the additional change observed in the spectrum of its acid solution.

SUMMARY

1. The stability, acid-solubility and ultraviolet absorption spectra of acridane and of 2-chloro-7-methoxyacridane have been measured under selected conditions.

2. The instability of these compounds, especially in acid media, makes measurements of solubility and absorption spectra of doubtful accuracy, and sometimes impossible.

3. Previous findings on the nature of mepacrine degradation products have been compared with those of the present work. The conclusions formerly reached still hold, but acridanes unsubstituted in the 5:10-positions can now be ruled out as excretion products from subjects on mepacrine therapy.

I should like to thank Prof. E. J. King for suggesting this investigation and for his continued interest in the work; Dr J. Kenyon, F.R.S. (Battersea Polytechnic, London, S.W. 11), for the hospitality of his department during one stage of this work; the Directors of Messrs May and Baker Ltd., for laboratory facilities given at another stage; Mr S. Bance, for the semimicro-analyses; and Mr W. R. Weedon for determining the absorption spectra.

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The Effect of Oxygen and of Cocarboxylase on the Formation of Citrate and α-Ketoglutarate by Pigeon Brain Homogenates

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The fact that vitamin B_1 is necessary for the relief of deficiency signs in the pigeon and for the disappearance of added or accumulated pyruvate *in vitro* and *in vivo* has always been an indirect argument for the decarboxylation of pyruvate to some C_2 fragment before its further oxidation, since aneurin pyrophosphate is concerned essentially with decarboxylation; thus in the animal an oxidative decarboxylation has appeared to be obligatory. In spite of this cogent evidence there has been no proof that, in fact, such a C_2 fragment is formed, which is clearly an unsatisfactory position. In a recent communication (Coxon, Liébecq & Peters, 1949) it was shown that citrate and α-ketoglutarate are produced during the oxidation of pyruvate by dialysed homogenates of pigeon brain, and that the yields of these compounds are increased greatly when fumarate is added. Though all difficulties in the application of the tricarboxylic acid cycle to brain tissue were not resolved, these findings pointed to the condensation of a derivative of pyruvate, or of pyruvate itself, with a four-carbon dicarboxylic acid along the lines

* With the technical assistance of Mr R. W. Wakelin.

postulated in that scheme, and, in agreement with this, oxaloacetate has since been shown to have an effect similar to that of fumarate. The formation of citrate and α-ketoglutarate through such a condensation should involve at some stage an oxidative decarboxylation and therefore oxygen should increase the yields of these substances. Evidence for the formation of a C_2 intermediate has been obtained by the demonstration that cocarboxylase as well as oxygen is necessary for a maximum production of these substances by brain tissue obtained from the vitamin B_1-deficient pigeon.

A preliminary account of this work was given to the Biochemical Society on 7 May 1949 (Coxon & Peters, 1949).

EXPERIMENTAL

Reagents. Oxaloacetic acid was kept as a solid which was dissolved in water and neutralized with NaOH immediately before use. Both Na pyruvate and oxaloacetic acid were prepared in this laboratory by Mr R. W. Wakelin. Adenosinetriphosphate (ATP) was obtained from Boots Pure Drug Co. as the Ba salt and was converted to the K or Na salt before use. Its purity was approximately 80%. Cocarboxylase was a commercial sample kindly supplied by Hoffman La Roche, to whom our best thanks are due. The buffer solution in all experiments was a mixture of KH_2PO_4 and Na_2HPO_4, the final concentration being 0·1 M and the pH 7·3.

Animals. The normal pigeons were maintained on a diet of equal parts of wheat and maize. The deficient birds were fed on polished rice until opisthotonus developed. As has been customary in this laboratory, on the first appearance of signs they received 200 mg. of glucose in 8 ml. of water. If the opisthotonus persisted after this treatment the bird was used immediately; otherwise it was allowed to survive until a relapse occurred. By this procedure more constant results are achieved when testing the effect of cocarboxylase *in vitro*.

Analytical methods. Citrate was determined by the method of Pucher, Sherman & Vickery (1936) as modified in this laboratory (Buffa & Peters, 1949) with the safeguards for specificity recommended by Breusch & Tulus (1947).

α-Ketoglutarate was determined by the method of Friedemann & Haugen (1943). In addition to the straightforward determination carried out according to their description, a second determination involving a modification of their procedure (Coxon, 1949, unpublished) was also performed. This modification minimized interference by the oxaloacetate and pyruvate present and consisted in the successive extraction of the mixture of 2:4-dinitrophenylhydrazones first with toluene and then with ethyl acetate. The second extract made in this way contained the greater part of the α-ketoglutarate compound with relatively little of the others and its composition provided a useful check on the figures obtained by the single-solvent technique. All readings were made with the Beckman spectrophotometer at 420 and 540 mμ.

Preparation of homogenates. The homogenates used throughout were 'dispersions' made by grinding the tissue in an ice-cold mortar with 0·9% KCl according to the technique of Banga, Ochoa & Peters (1939*b*) and were in all cases dialysed against 0·45% KCl at 2° for about 4 hr. Similar results could be obtained by centrifuging in the cold instead of dialysing.

Incubation of tissue and substrates. Solutions containing the requisite amounts of pyruvate, $MgCl_2$ and ATP were introduced into the main compartments of Warburg flasks while oxaloacetate (or fumarate) was placed in the side bulbs. The flasks were kept in ice until the homogenate, which was mixed with buffer after removal from the dialyser, had been added, and while CO_2-absorption papers were placed in the centre wells. Where it was desired to induce O_2 lack, the flasks were connected, while still surrounded with ice, to manometers which were then flushed with N_2 for 5–10 min. After this period they were transferred to the water bath (38°) for temperature equilibration and the passage of N_2 was continued for 5 min. more. Temperature equilibrium was assumed to have been established after a further 5 min., at the end of which time the taps were closed and the contents of the side bulbs were tipped into the main compartments of the flasks. The flasks containing air were treated similarly as regards conditions of temperature. Incubation (in the thermostat) was carried out for 30 min. in most of the experiments; trichloroacetic acid to a final concentration of 5% was then added, and, after allowing 15 min. for the protein precipitate to coagulate, this was separated and the filtrate stored in a refrigerator to await analysis.

RESULTS

Optimal concentrations of substrates

Owing to the limitations of the analytical method it was necessary to restrict the amount of pyruvate and oxaloacetate added so that their total concentrations in the final filtrate would not mask the determination of the α-ketoglutarate. These restrictions resulted in the production of submaximal quantities of citrate (and presumably of α-ketoglutarate), but the pattern of behaviour in the different conditions under test was the same whether large or small total concentrations were employed, as may be seen from Table 1. It was also found in preliminary experiments that large amounts of oxaloacetate tended to produce inhibition of oxygen uptake instead of the stimulation which was constantly observed with the smaller amounts. This point is illustrated in Table 2. A similar effect was described by Potter, Pardee & Lyle (1948) using

Table 1. *Effect of differing amounts of pyruvate and oxaloacetate*

(Each bottle contained in 3·0 ml. of 0·1 M-phosphate buffer, pH 7·3, Mg^{++}, 4·0 μmol., ATP, 1·0 μmol., together with pyruvate and oxaloacetate as stated and approx. 200 mg. of tissue. Incubation 30 min. at 38°.)

Substrate added (μmol.)		Citrate found (μmol.)	
Pyruvate	Oxaloacetate	In N_2	In air
10	10	0·67	1·31
30	20	1·75	3·04

Table 2. *Effects on oxygen uptake of differing concentrations of oxaloacetate*

(Each bottle contained in 3·0 ml. of 0·1 M-phosphate buffer, pH 7·3, Mg^{++}, 4·0 μmol.; ATP, 1 μmol.; together with pyruvate and oxaloacetate as stated and approx. 200 mg. of tissue. Temp., 38°.)

	O_2 uptake (μl. in 30 min.)			
Exp.	Pyruvate alone (10 μmol.)	Pyruvate (10 μmol.) + oxaloacetate (10 μmol.)	Pyruvate alone (30 μmol.)	Pyruvate (30 μmol.) + oxaloacetate (30 μmol.)
1	87	112	—	—
2	—	—	135	105
3	—	—	191	178

kidney homogenates. As a result of these findings we used 10 μmol./bottle of both pyruvate and oxaloacetate in most of our experiments.

Effect of oxygen on normal brain

The starting point of the studies on avitaminous brains was the observation that the production of citrate and α-ketoglutarate was very markedly reduced in the absence of oxygen. Some data illustrating this phenomenon are given in Table 3.

Table 3. *Effect of oxygen on production of citrate and α-ketoglutarate by brain homogenate from normal pigeons*

(Each bottle contained in 3·0 ml. of 0·1 M-phosphate buffer, pH 7·3, Mg^{++}, 4·0 μmol.; ATP, 1 μmol.; together with pyruvate and oxaloacetate as stated and approx. 200 mg. of tissue. Incubation 30 min. at 38°.)

	Citrate found (μmol./bottle)		α-Ketoglutarate found (μmol./bottle)	
	In N_2	In air	In N_2	In air
After equilibration	0·5	1·3	Nil	About 0·2
30 min. later	0·6	1·9	About 0·2	1·4

Effects of oxygen and of cocarboxylase on avitaminous brain

The effects of oxygen and of cocarboxylase are set out together in Tables 4 and 5, since in a number of instances they were tested simultaneously on the same brain. Tables 4 and 5 show that these two factors both increased the production of citrate and α-ketoglutarate; where the experiments are similarly numbered in the two tables this means that the same filtrates were analysed for the two products.

Table 4. *Effects of oxygen and of cocarboxylase on the production of citrate by dialysed homogenates of pigeon brain*

(Each bottle contained pyruvate, 10 μmol.; $MgCl_2$, 4 μmol.; ATP, 1·0 μmol.; homogenate equivalent to 200 mg. of fresh brain tissue, together with 10 μmol. of oxaloacetate in the side bulb. Bath at 38°.)

	Citrate formed in 40 min. (μmol./bottle)			
Exp. no.	In N_2	In N_2 with cocarboxylase (10 μg.)	In air	In air with cocarboxylase (10 μg.)
1	0·38	0·68	0·40	0·90
2	0·48	0·84	0·41	1·10
3	0·50	0·83	1·20	1·50
4	—	—	0·56	1·00

Table 5. *Effect of oxygen and of cocarboxylase on the production of α-ketoglutarate by dialysed homogenates of pigeon brain*

(Each bottle contained pyruvate, 10 μmol.; $MgCl_2$, 4 μmol.; ATP, 1·0 μmol. and homogenate equivalent to 200 mg. of fresh brain tissue, together with 10 μmol. of oxaloacetate in the side bulb. Incubation 40 min. at 38°.)

	α-Ketoglutarate formed (μmol./bottle)			
Exp. no.	In N_2	In N_2 with cocarboxylase (10 μg.)	In air	In air with cocarboxylase (10 μg.)
1	0·55	0·73	—	—
2	0·0	0·13	0·80	2·20
3	0·0	0·55	1·37	1·78
4	—	—	0·20	0·94

Table 6. *Effect of cocarboxylase on the production of α-ketoglutarate and of citrate by dialysed homogenates of pigeon brain in presence of fumarate*

(Each bottle contained pyruvate, 10 μmol.; $MgCl_2$, 4 μmol.; ATP, 1 μmol. and homogenate equivalent to 200 mg. of fresh brain, together with 10 μmol. of Na fumatate. Incubation 30 min. at 38°.)

Cocarboxylase added (μg./bottle)	Citrate found (μmol./bottle)	α-Ketoglutarate found (μmol./bottle)
0	0·6	0·0
10	1·3	1·5

Effect of cocarboxylase with fumarate replacing oxaloacetate

Since fumarate is presumed, on the basis of previous work, to increase the yield of citrate and α-ketoglutarate by supplying oxaloacetate, and since this action would involve an oxidation, there seemed no reason to try anaerobic experiments using fumarate; however, the favourable effect of

cocarboxylase on the aerobic production of citrate and α-ketoglutarate in one instance is shown in Table 6.

DISCUSSION

Much previous work from this laboratory (Banga, Ochoa & Peters, 1939*a*) has shown that cocarboxylase acts in the animal as coenzyme for the decarboxylating step in the degradation of pyruvate. (Another suggestion by Krebs & Eggleston (1940) that it may catalyse the carboxylation of pyruvate to form oxaloacetate does not appear to have been substantiated.) We therefore consider that the evidence put forward in the present paper, while confirming earlier suggestions (Coxon *et al.* 1949) regarding the connexion between the oxidation of pyruvate and the formation of citrate and α-ketoglutarate in brain homogenates, also demonstrates directly that an oxidative decarboxylation of pyruvate with the production of some two-carbon fragment must precede its condensation with oxaloacetate and subsequent participation in a tricarboxylic acid cycle. This follows from the finding that the addition of cocarboxylase and oxygen to suitable preparations increased the yields of citrate and α-ketoglutarate. For pigeon brain, it appears to dispose of the suggestion (Wood, Werkman, Hemingway & Nier, 1942; Breusch, 1943) that the first product of condensation is a seven-carbon compound, for if such a compound, of the type of pyruvofumarate, were formed (that is, if the oxidative decarboxylation which we have demonstrated occurred after instead of before the condensation) then it would be expected that the hypothetical seven-carbon compound would accumulate in the blood of animals suffering from aneurin deficiency whereas in fact, within the limits of spectrophotometric analysis, it is pyruvate which appears to accumulate in this condition.

The small amounts of citrate and α-ketoglutarate which are formed anaerobically call for some comment. Breusch (1939) reported the anaerobic formation of citrate in small amounts by minced brain (from pigeons and cats) incubated with pyruvate and oxaloacetate. Krebs, Eggleston, Kleinzeller & Smyth (1940) also recorded that considerable amounts of α-ketoglutarate and smaller quantities of citrate could be formed anaerobically from oxaloacetate by minced sheep brain. We have not made determinations of malate in our experiments, but we suggest that such citrate as is formed by our homogenates under anaerobic conditions arises by a process involving an oxidative decarboxylation coupled with a reduction, probably of oxaloacetate, to malate; this would explain the effect of cocarboxylase in augmenting the yield to some extent even in the absence of molecular oxygen.

Since the preliminary report of our findings was given to the Biochemical Society, Stern & Ochoa (1949) have adduced evidence that in liver, at all events, citrate may be formed from acetate plus oxaloacetate without the intervention of *cis*-aconitate or *iso*citrate, while Potter & Heidelberger (1949) have published results implying that much of the reasoning which had been criticized earlier by Ogston (1948), but which had formed the basis for the relegation of citrate to the role of a side product of the tricarboxylic acid cycle, was actually invalid. So far as pigeon-brain homogenates are concerned, the assumption that the main pathway is through *cis*-aconitate with citrate arising by a side reaction affords a readier explanation of the fact that their oxygen uptake is stimulated by the former, but not by the latter (Coxon *et al.* 1949). Nevertheless, in the absence of reliable quantitative data on the readiness with which *cis*-aconitate, *iso*citrate and citrate are interconverted by our system and on the kinetics of the process it would be unjustifiable to propose a rigid concept of the sequence of events at this point in the reaction scheme. Moreover, the synthesis of citrate by Stern & Ochoa's (1949) liver preparation was accomplished anaerobically so that the relationship of their results to the behaviour of our brain dispersion is far from clear. The 'citrogenase' reaction described by Breusch (1943), in which a β-keto acid condenses with oxaloacetate to form citrate, is stated by him to be independent of oxygen tension; to bring pyruvate into that reaction he postulates its conversion to acetate, two molecules of which combine to give one of acetoacetate, a process which would involve an oxidative decarboxylation. However, Breusch (1948) states that 'citrogenase' is not present in great quantity in brain and in the absence of positive evidence in favour of it we see no reason to prefer this scheme to the more usually accepted one.

SUMMARY

1. Both oxygen and aneurin pyrophosphate (cocarboxylase) increase the formation of citrate and α-ketoglutarate from pyruvate and oxaloacetate by homogenates of pigeon brain.

2. It is concluded that in this tissue an oxidative decarboxylation of pyruvate to some C_2 fragment precedes the condensation with oxaloacetate.

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The Reactivity of the Iminazole Ring in Proteins

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The sulphydryl, disulphide (cf. Anson, 1945), phenolic (Crammer & Neuberger, 1943) and amino groups (Porter, 1948) of proteins show varying degrees of reactivity in the native molecule. On denaturation of the protein by any means, these groups, with very few exceptions, become capable of reacting completely with the several reagents examined. This has been interpreted as suggesting the existence, in the native protein, of labile bonds or steric factors which are altered on denaturation. It has now been found, using the reagent 1:2:4-fluorodinitrobenzene (FDNB), that the iminazole rings of the histidine residues of β-lactoglobulin are less reactive in the native than in the denatured protein. Other proteins examined did not show this difference in reactivity. In insulin the iminazole group was reactive in the native molecule, but on heat precipitation, or fibrous insulin formation, almost half the residues became unreactive. Fibrous insulin, superficially resembling denatured protein, is believed to be a long-chain polymer of the insulin molecule (Waugh, 1948), and it would appear that in this case intermolecular association, rather than intramolecular rearrangement is responsible for the change in reactivity of the histidine residues.

MATERIALS

Haemoglobins. These were prepared as described by Porter & Sanger (1948).

Bovine serum albumin. A sample prepared by cold ethanol fractionation was obtained from Armour Ltd., Chicago, U.S.A.

β-Lactoglobulin. This was a crystalline sample given by Prof. Linderstrøm-Lang.

Ovalbumin. This was a five-times recrystallized sample obtained by Prof. A. C. Chibnall from Prof. R. K. Cannan.

Insulin. Crystalline ox insulin from Boots Pure Drug Co. Ltd. was used. Heat-precipitated insulin was prepared by keeping a 3% solution of insulin in 0·1 N-H_2SO_4 at 95° for 30 min. (du Vigneaud, Sifferd & Sealock, 1933). The precipitate was centrifuged and washed with distilled water. Fibrous insulin was prepared as an opalescent gel according to Waugh (1948).

METHODS

The conditions of denaturation and of condensation of FDNB with proteins have been described by Porter (1948).

Estimation of unreactive histidine residues in proteins

It was suggested by Sanger (1945) that FDNB did not form a stable compound with the iminazole ring of histidine, but it has since been found that a hydrolysate of dinitrophenyl-(DNP-) globin contains no free histidine. Dr Sanger has re-examined the reaction of FDNB with *N'*-acetylhistidine and found that a stable crystalline compound, m.p. 221° (decomp.), is in fact formed. After removal of the acetyl group the colourless crystalline DNP derivative, m.p. 280° (decomp.), has a nitrogen content corresponding to mono-DNP-histidine and its probable structure is 1-(2′:4′-dinitrophenyl)-4(5-)iminazole-α-aminopropionic acid (im-DNP-histidine). On acid hydrolysis it did not give rise to histidine (Sanger, unpublished).

The condensation of FDNB with protein is carried out in aqueous ethanol at room temperature; the reaction is so rapid that it precedes and prevents ethanol denaturation of

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β-lactoglobulin and probably of other proteins (Porter, 1948). A comparison of the reactivity with FDNB of the iminazole ring in native and denatured proteins should give some indication of the condition of this group in the native molecule. As im-DNP-histidine is a colourless compound a simple method of fractionation and colorimetric estimation was not possible. The amount of histidine unable to react with FDNB was therefore estimated directly in the hydrolysate of the DNP-protein.

Experimental procedure. The procedure was as follows: (1) The protein was allowed to react with FDNB and the protein content of the air-dried DNP-protein calculated from amide estimations as described previously (Sanger, 1945). (2) A weighed amount of air-dried DNP-protein was hydrolysed for 24 hr. in boiling 5·7 N-HCl. Excess HCl was removed by repeated concentration *in vacuo*; the residue was neutralized to pH 4·5 with N-NaOH and made up to a known volume with 0·2 M-acetate buffer pH 4·5. Owing to the small amounts of free histidine remaining in DNP proteins it was necessary to work with concentrated solutions; thus, a hydrolysate from 200 mg. would be made up to 10 ml. and 2·5 ml. used per estimation. A precipitate frequently appeared on neutralization but did not interfere. (3) Histidine was estimated manometrically using the specific decarboxylase (Gale, 1945). The products of hydrolysis of DNP proteins did not affect the activity of the enzyme, but they would, of course, hinder colorimetric estimation. Satisfactory dulplicates were obtained and all the results quoted are the mean of two or more assays.

RESULTS

In some of the proteins examined, FDNB condensed almost quantitatively with the iminazole ring and only a small blank value equivalent to 2 or 3 % of the original histidine remained. As indicated in Table 1, native lactoglobulin was an exception; 50 % or two of the four histidine residues per molecule did not condense with FDNB. After denaturation by any of the three methods tried, all the histidine residues of lactoglobulin reacted with FDNB.

The result with human haemoglobin (Table 1) is of interest because it might be expected that, if haem is attached to the iminazole ring, four of the histidine residues (12·5 % of the total) would fail to react with the FDNB. As the linkage of haem to globin is very labile, the reaction was carried out at −3° for 30 hr., but again the histidine residues reacted almost quantitatively with FDNB. Though no haem was removed when the DNP-haemoglobin was washed with water, ethanol and ether, subsequent spectroscopic examination, kindly carried out by Miss J. Keilin, showed that a considerable amount of free haematin and haem were present together with haemochromogen. The haem-globin bond had been broken and no conclusion as to its nature could be drawn.

Insulin proved exceptional in that a small but significant fraction of the histidine did not react when crystalline insulin was used and a much larger proportion was unreactive in heat-precipitated and fibrous insulin (Table 2). Throughout the experiments with insulin, the sodium bicarbonate, FDNB and ethanol-water concentrations were the same, the only variable being the condition of the insulin. The sodium bicarbonate concentration used was 4 % not 10 % (w/v of aqueous protein solution) as in the other experiments, because the higher concentration throws insulin out of solution and is not necessary to keep the mixture alkaline throughout the reaction.

Table 1. *Reaction of histidine residues of proteins with* FDNB

Protein	Histidine content (N as % of total N)	Histidine content after condensation with FDNB. (N as % of total N of original protein)	Percentage of unreactive histidine residues
Bovine serum albumin	7·24 (*a*)	0·36	5·2
Native β-lactoglobulin	2·85 (*b*)	1·46	51·0
Heat-denatured β-lactoglobulin	2·85	0·04	1·4
Guanidine-denatured β-lactoglobulin	2·85	0·04	1·4
Ethanol-denatured β-lactoglobulin	2·85	0·07	2·0
Native ovalbumin	4·1 (*b*)	0·12	2·9
Heat-denatured ovalbumin	4·1	0·10	2·5
Horse globin	13·5 (*b*)	0·34	2·5
Human haemoglobin	13·1 (*c*)	0·5	3·9

References. (*a*) Brand (1946); (*b*) Macpherson (1946); (*c*) Roche (1936).

Table 2. *Reaction of insulin with* FDNB

Conditions of insulin in reaction with FDNB	Unreactive histidine (as % of total histidine)	No. of unreactive groups/submolecule of wt. 12,500
Solution of insulin (0·1 %)	7·5	0·30
Solution of insulin (1 %)	9·0	0·36
Solution of insulin (3 %)	10·1	0·42
Solution of insulin (5 %) in 6 M-urea	11·2	0·45
Heat-precipitated insulin in suspension	44·0	1·75
Fibrous insulin in suspension	45·0	1·80

DISCUSSION

β-Lactoglobulin

Under the conditions of the reaction, the histidine residues of serum albumin, egg albumin and haemoglobin are able to condense with FDNB whether the proteins have been denatured previously or not, but in β-lactoglobulin only two of the four residues are available to the reagent, prior to denaturation. In view of the rapidity of the reaction of FDNB with β-lactoglobulin (Porter, 1948), and the failure of a considerably increased reaction period, in the presence of excess reagents, to affect the number of reacting iminazole groups, it is probable that there is a qualitative distinction between the reactive and unreactive groups. It is suggested that, in this native protein, histidine is an additional example of a residue whose reactivity is limited by the specific structure of the molecule.

Insulin

In native insulin there is a persistent content of unreactive histidine which appears to increase slightly with concentration (Table 2); the presence of 6 M-urea has no effect and it has equally little effect on the biological activity or crystallizing power of the insulin. These values of unreacted histidine, about 10 % of the total, are significantly higher than the blank values found for other proteins. As a number of different preparations of DNP-insulin were used, some of which had been made by allowing the reaction to continue for a considerably greater time than the usual 2 hr., it is most unlikely that the free histidine arose from a fortuitous failure of the reaction to go entirely to completion.

It has been postulated that the basic units of insulin have a molecular weight of about 12,000 (cf. Sanger, 1949), and it is believed that these units are associated in solution, reaching, under optimal conditions of pH and concentration, maximal aggregates of four units. At 3 % concentration and pH 8·5, insulin would be such an aggregate of four units, but under these conditions only 0·4 residues of histidine are unreactive per unit, and it is therefore clear that there can be no connexion between this association and the lack of reactivity of the histidine residues. In contrast, the lack of reactivity of nearly two out of four of the iminazole groups in the submolecule of fibrous or heat-treated insulin is due, probably, to the association of the units by endwise attachment. The insolubility of heated insulin is unlikely to explain the altered reactivity of the protein as amino groups react completely in the same sample and experience with β-lactoglobulin and other proteins suggests that the reactivity of all groups increases rather than decreases when the soluble native material becomes denatured and insoluble. Waugh (1948) has postulated that the formation of long fibres is due to the presence of two 'active centres' in each unit. If this is accepted, it could be assumed that two histidine residues become unreactive because they are part of such combining centres, or are sufficiently close to these centres for increased steric hindrance to prevent access of the FDNB molecule. The reactivity of the N^1 and N^5 amino groups (six/submolecule) is unaffected by heat precipitation. This evidence supports the view that the combining centres are localized on the surface of the molecule.

In pointing out that aggregation occurred only at low pH, Waugh (1948) suggested that suppression of the ionization of the acidic groups might be essential to reduce electrostatic particle repulsion. By similar reasoning it seems unlikely that the two iminazole residues involved are situated on the two combining centres, since this would lead to the association of two similarly charged groups. It is more probable that both of the iminazole groups involved are in one combining centre, the second centre being of different composition. Such asymmetry of the molecule would be in keeping with the finding of Sanger (1949) that all the basic amino-acid residues are in the two peptide chains which have terminal phenylalanyl residues, the other two peptide chains with terminal glycyl residues containing no basic amino-acids.

If the blocking of the iminazole residues in heat-precipitated or fibrous insulin is due to the polymerization of submolecules, the small amount of unreactive material in crystalline insulin is perhaps explicable by the assumption of contamination of the protein by a little polymerized material. Seeding experiments carried out by Waugh showed that such contamination could not occur at acid reaction, but in neutral or alkaline solution it may not be impossible. This, or the presence of some other bound impurity, seems to be the only explanation of the results obtained with the crystalline material.

SUMMARY

1. 1:2:4-Fluorodinitrobenzene reacts with the iminazole ring of N^1-acetyl-histidine to give a compound which does not yield histidine on acid hydrolysis.

2. With the aid of this reaction it has been found that two of the four histidine residues in β-lactoglobulin fail to react with fluorodinitrobenzene unless the protein is first denatured. In ovalbumin, serum albumin, haemoglobin and globin the histidine residues react fully whether the proteins are denatured or native.

3. In crystalline zinc insulin 0·3–0·4 histidine residues of the four present per submolecule fail to react, and after heat precipitation or conversion to fibrous insulin, 1·8 residues become unreactive. The

significance of these findings in relation to fibre formation is discussed.

4. Unsuccessful attempts to use this reaction to establish the mode of linkage of haem to globin in native haemoglobin are described.

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Studies on the Synthesis of Lactose by the Mammary Gland

I. PRECURSORS

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The main object of our work is to throw some light on the intermediary metabolism of the mammary gland, with particular reference to the mode of lactose synthesis. Although a number of workers, using a variety of experimental techniques, have made an approach to this problem, all studies hitherto reported have been primarily concerned with defining the initial blood precursors of the milk sugar, and there is little information on the detailed mechanism of this synthesis. *In vivo* experiments, in which the concentrations of possible precursors in simultaneously drawn samples of arterial and mammary venous blood were compared, have demonstrated a large uptake of glucose when blood passes through the active mammary gland (for references see Folley, 1940, 1949), and it is regarded as reasonably certain that this uptake is far in excess of the energy requirements of the tissue, and is, in fact, an indication that glucose is a precursor of lactose.

This conclusion has been confirmed and amplified by *in vitro* studies on surviving mammary tissues, notably by Grant (1935) and Knodt & Petersen (1945). Grant (1935) succeeded in demonstrating lactose synthesis from glucose by tissue slices from the lactating glands of guinea pigs killed 2–5 days after parturition, and was able to obtain a quantitative estimate of the synthesis by using a lactose-fermenting yeast, *Saccharomyces fragilis*. Knodt & Petersen (1945) claim to have shown lactose synthesis by tissue slices from the lactating udders of cows killed at slaughter, and in their work glucose, glycogen and maltose were found to be equally effective substrates. Lactic acid, on the other hand, was ineffective, a result contrasting with earlier claims based on arterio-venous studies by Graham (1937). Knodt & Petersen (1945) used fermentation techniques (Scott & West, 1936) in determining lactose and, like Grant (1935), were dependent on changes in reduction values following fermentation for their estimation of lactose formed.

It seemed to us that a first step in our work should be a reinvestigation of these findings, using methods for the estimation and detection of lactose which could be regarded as more specific for this sugar than methods ultimately dependent on reduction measurements. This paper gives an account of this initial study.

EXPERIMENTAL

Reagents. Glycogen was prepared from rabbit livers by extraction with trichloroacetic acid and subsequent precipitation with 50% (v/v) ethanol, 80% (v/v) acetic acid and 50% (v/v) ethanol in that order (Good, Kramer & Somogyi, 1933; Bell & Young, 1934). *s*-Collidine was purified according to Partridge (1948).

Sources of tissues. The collection of cow udders from the local abattoir conformed as closely as possible with the procedure adopted by Knodt & Petersen (1945). Glands showing a high degree of milkiness on removal were selected and brought without special cooling direct to the laboratory. The time from slaughter to the start of an experiment was normally 1·0–1·5 hr.

Guinea pig glands were obtained from animals in our own colony. Parturient does were suckled normally by their litters and killed in active lactation on the sixth day post-partum. In these experiments the incubations of tissue started within 30 min. of killing by neck dislocation.

Preparation of tissues. Both cow and guinea pig glands were prepared in the same way. Tissue slices were cut with a Stadie & Riggs (1944) microtome from small blocks of tissue immersed in Krebs Ringer phosphate (KRP) buffered at pH 7·4 (Krebs & Henseleit, 1932; Umbreit, Burris & Stauffer, 1945). The microtome inset had been ground to cut slices 0·5 mm. thick, but owing to the compressibility of mammary tissue the actual thickness of the slices used was variable, ranging from 0·5 to 1·0 mm. The slices were quickly washed with three changes of KRP, blotted on filter paper, washed once more, blotted again and added in equal portions to the incubating flasks. By this treatment the elimination of any preformed milk in the gland was ensured. Guinea pig glands normally gave sufficient material for four to five flasks.

On a few occasions experiments were performed using mammary gland extracts. These were prepared by homogenizing glands in ice-cold KRP in a tissue homogenizer (Folley & Watson, 1948), centrifuging the suspensions obtained and filtering the supernatants through sintered glass filters.

Incubations. These were carried out in loosely stoppered 150 ml. conical flasks, in KRP buffered at pH 7·4, with air as the gaseous phase. The flasks were shaken for 4 hr. in a water bath at 37°. Substrates were initially present in a concentration of 0·5% (w/v), and the tissues, on a dry-weight basis, in a concentration of roughly 3% (w/v). The volumes were usually 20 ml. for the experiments with cow glands and 15 ml. for those with guinea pig tissue. Control experiments with tissue alone in the absence of substrates were included in all experiments.

Pre-estimation procedure. At the end of the incubation period the mixtures were centrifuged and the solutions fermented for 1 hr. at 37° with washed suspensions of either brewer's or baker's yeast. The latter was used when glucose had been the only substrate. The fermentations were needed to clear the solutions of the substrates (glucose, maltose), or to remove any maltose that might be formed by amylolytic breakdown of glycogen; they also served to destroy a part of the lower dextrins which were formed when glycogen was used as a substrate. This last point will be more fully considered under RESULTS. In many of the guinea pig experiments, however, the glycogen mixtures were treated slightly differently in order to ensure a more complete removal of dextrins. An equal volume of ethanol was added to precipitate glycogen, and the solutions were filtered and evaporated to dryness on a water bath; after redissolving the residues in water, salivary amylase was added and the solutions incubated for 1 hr. at 37°; fermentation was then carried out with brewer's yeast as outlined above.

After centrifuging the fermented solutions, the supernatants were prepared for estimation by methods outlined by Malpress & Morrison (1949*a*). With cow tissue, $Zn(OH)_2$ was used as a deproteinizing agent whenever the substrate had been glucose or maltose; in the guinea pig experiments, however, almost all the solutions were precipitated with ethanol, thus giving a greater uniformity to the pre-estimation procedures adopted for solutions from the experiments with glucose, maltose and glycogen.

Dry weight determinations were made by drying the washed residues from the centrifuged mixtures at 100° overnight.

Estimations. An approximation to the lactose content of the fermented and prepared solutions was first obtained by measuring reduction values by the method of Somogyi (1945) on the equivalent of 1 ml. of the original mixture; these results were then compared with those given by the more specific method for lactose of Malpress & Morrison (1949*a*), usually carried out on the equivalent of 4 ml. of the original mixture. Throughout this paper, results are given as mg. of 'lactose' formed per g. dry weight of tissue, during a 1 hr. incubation period.

Chromatographic analysis of the guinea pig incubates was made on samples of the solutions prepared as above, and deionized by pyridine extraction (Malpress & Morrison, 1949*b*). The experimental technique of Partridge (1948) was used, with only minor modifications. Sugar solutions from any one series of experiments were concentrated to the same final volume before applying to the paper. The final concentration of the sugars present thus varied according to the initial substrate and the degree of synthesis obtained; the usual apparent concentrations, judged from the estimation values, ranged from 0·5 to 4·0%, but, as will be seen, the actual lactose concentrations, especially those from the glycogen experiments, were in fact much lower.

It was found that, under our conditions, the R_F value for a given sugar was not always the same from day to day, and it has been our practice to run control solutions of pure sugars on the chromatograms for direct comparison with the sugars in the experimental solutions (cf. Jermyn & Isherwood, 1949).

RESULTS

Cow glands

After several experiments had been performed on these glands with glucose, glycogen and maltose as substrates, it became clear that they did not provide suitable material for studies on lactose precursors. The results obtained, however, are given here for their interest in relation to the contrary findings of Knodt & Petersen (1945).

Glucose experiments. The results of ten experiments showed that after incubating mammary tissue from cow udders with glucose, there was a slight increase in non-fermentable sugar reduction values in all except one instance, where there was no change; on the other hand, there was no evidence of lactose synthesis by the more specific methylamine method, and it was clear that any increase in the reduction values was not due to the formation of this sugar. It was repeatedly shown that amounts of lactose equivalent to those apparently determined by reduction were readily assessed by the methylamine method when added to the fermented and prepared mixtures, and also that small amounts of lactose added to mixtures at the start of an experiment were recovered in nearly theoretical yields. The ranges and mean values of 'lactose' synthesis given by cow tissue in the presence of glucose are given in Table 1.

Glycogen experiments. It was at first thought that lactose was being rapidly synthesized when glycogen was present as substrate. By reduction, seven experiments showed a mean synthesis of 8·5 mg. per g. dry weight of tissue in the unfermented mixtures after precipitation of glycogen, whilst by the methyl-

amine method a corresponding value of 21·0 mg. was obtained; the mean synthesis in all the eleven experiments in which lactose was estimated by the methylamine method was 19·9 mg. The lack of agreement between the figures given by the two methods clearly suggested that a substance, or substances, other than lactose was being estimated by the methylamine method; but the presence of maltose, the only other

Table 1. *Formation of 'lactose' in solutions from glucose incubates (cow)*

Method	Mean (mg./g. dry wt.)	Range (mg./g. dry wt.)
Reduction	0·4	0 –1·4
Methylamine	–1·0	–2·1–0·2

likely substance known at that time to give the methylamine reaction, could scarcely be held to account for the discrepancy, since for both methods the calibration curves for this sugar are very close to those of lactose. Fermentation with brewer's yeast, however, under conditions in which comparable amounts of maltose added to the mixtures were fermented in 15–20 min., showed that reducing substances, capable of giving the methylamine reaction, and far more slowly fermentable than maltose, were present in these solutions. A typical fermentation experiment gave the results recorded in Table 2. In one such experiment both the reducing power and the methylamine value were negligible after fermentation for 4 hr. with brewer's yeast.

Table 2. *Evidence of slowly fermentable products in solutions from glycogen incubates (cow)*

Time of fermentation (hr.)	Reducing value (as mg. of 'lactose')	Methylamine value (as mg. of 'lactose')
0	12·0	23·0
1	5·3	12·3
2	4·6	10·0
4	2·0	3·8

Further work on the nature of this fermentable material showed that after hydrolysis with N-hydrochloric acid the reducing power was 2–7 times greater than that of the unhydrolysed solutions; that a similar substance could be prepared by incubating starch with mammary tissue, glycogen with liver tissue, or either of these substrates with salivary amylase when the enzyme action was stopped at the achromic point; and finally that salivary amylase if allowed to act upon a preparation of the slowly fermentable material converted it into a reducing substance, readily fermentable by brewer's yeast, which was apparently maltose.

We concluded from these experiments that the 'lactose' formed in the glycogen experiments was in reality a variable mixture of lower dextrins and maltose, produced by amylolytic action; the dextrins were soluble in 50 % (v/v) ethanol, since this treatment was used in precipitating glycogen from the solutions, and also, were capable of giving a strong methylamine test.

Maltose experiments. No evidence of the formation of lactose from maltose was found, an increase in reduction values being offset by variable negative or slightly positive methylamine figures. The results thus closely resembled those given by glucose.

Guinea pig glands

Glucose incubates. Unlike the cow glands, guinea pig tissue gave marked evidence of the formation of lactose from glucose when the synthesis was judged by the increase in reducing power of the solutions and also by the methylamine colour reaction. As will be noted later, this evidence was borne out by chromatographic analysis. The results of twelve experiments are given in Table 3. Experiments in which fermentation was prolonged for periods up to 4 hr. gave results from which it may be concluded that all fermentable material had been removed from the solutions recorded in Table 3.

Usually the ratio 'lactose by reduction/lactose by methylamine' (R/M), was greater than one, a result which we interpret as indicating the greater specificity of the methylamine method; occasionally, however, the ratio fell below unity indicating that in some cases the methylamine method was not proving wholly specific for lactose in these estimations on glucose mixtures. We may note, however, that the only substances, other than lactose, at present known to give the methylamine reaction are maltose and cellobiose, both disaccharides with a 1:4-glucosidic linkage, and the lower dextrins already commented on above. None of the other common products of glucose metabolism gives the reaction, nor have we found the reaction with other tissues incubated in the presence of glucose. Since, therefore, the non-lactose reactant in these experiments (*a*) clearly

Table 3. *Formation of 'lactose' in solutions from glucose, glycogen and maltose incubates (guinea pig)*

Substrate	'Lactose' by reduction (R) (mg./g. dry wt.) Mean	'Lactose' by reduction (R) (mg./g. dry wt.) Range	'Lactose' by methylamine (M) (mg./g. dry wt.) Mean	'Lactose' by methylamine (M) (mg./g. dry wt.) Range	R/M Range
Glucose	7·2	1·7–13·0	5·3	1·0–10·0	0·5–2·8
Glycogen	2·9	1·3– 5·1	7·2	3·8–16·1	0·1–1·2
Maltose	0·6	–0·4– 1·6	0·4	–0·6– 1·2	—

derives from glucose (there was no methylamine colour in control mixtures) and (*b*) only appears when lactose is being synthesized (under similar conditions cow glands failed to give any colour at all), we feel justified in associating it closely with the metabolic pathway of lactose synthesis. We also feel justified in suggesting tentatively that the methylamine values give, in fact, a measure of lactose plus its immediate, and probably non-reducing, precursor (possibly a sugar-1-phosphate), and that in any case they afford a more useful indication of the synthetic activity of the gland than do the changes in reducing power.

Glycogen experiments. Four-hour fermentations showed that slowly fermentable dextrins were present after incubation when glycogen was the substrate. They were even less readily fermentable than those formed by cow tissue. Accordingly, prefermentation treatment with salivary amylase was adopted as routine for these solutions in an attempt to remove the dextrins completely (see Experimental section). The results of six experiments on glycogen mixtures given this revised treatment are shown in Table 3. Analysis by paper chromatography gave direct proof of the presence of small amounts of lactose in these clarified solutions, but it is reasonably certain that in most of these the high methylamine values were largely due to the incomplete removal of dextrins by ptyalin action, a theory supported by the low R/M ratios and by chromatographic evidence (see below).

Maltose experiments. No evidence was obtained for the formation of lactose from maltose by the guinea pig mammary gland. The results of three experiments are included in the data given in Table 3.

Synthesis from lactate. A single experiment in which mammary tissue from a guinea pig was incubated with glucose, glucose plus sodium lactate (lactate 0·5% w/v), and sodium lactate alone (lactate 0·5% w/v), showed the inability of the gland to form lactose from lactate, or to utilize this substrate in increasing lactose formation from glucose (Table 4).

Table 4. *Effect of lactate upon 'lactose' synthesis by the guinea pig gland*

Substrate	'Lactose' by reduction (mg./g. dry wt.)	'Lactose' by methylamine (mg./g. dry wt.)
Glucose	13·0	7·3
Glucose + Na lactate	11·3	7·6
Na lactate	0·4	1·2

Use of extracts

No lactose synthesis could be shown by extracts of any gland, cow or guinea pig, whatever the substrate used. This is in accord with the work of Grant (1936).

Chromatography

The formation of lactose when either glucose or glycogen were the substrates for slices of active guinea pig mammary gland was confirmed by the analytical methods of paper chromatography. In four out of five comparisons the lactose-reduction spot from the glucose mixture was much more intense than that from the glycogen experiment from the same gland. The exception showed a greater synthesis from glycogen, but the abnormality seems to have been a very poor lactose yield from glucose, for some unknown reason, rather than an enhanced yield from glycogen.

Comparison between experiments with glycogen and maltose with the same glands showed formation of lactose from glycogen but none from maltose.

Although quite definite, the lactose spots from glycogen experiments were frequently weak, thus bearing out the significance already given to the high methylamine values and low R/M ratios quoted in Table 3. The nature of the residual colour-developing substances in these solutions was made more certain when paper chromatograms of the hydrolysed solutions were prepared. These showed a very intense glucose spot which again strongly suggested the presence of some lower dextrins in the unhydrolysed mixtures.

DISCUSSION

Our results with cow mammary gland tissue are of interest in that they disagree with the findings of Knodt & Petersen (1945); whereas our work leads us to the conclusion that neither glucose, glycogen nor maltose are suitable substrates for lactose synthesis by cow mammary glands, which were apparently lactating and were collected direct from slaughter, Knodt & Petersen (1945) claimed lactose synthesis from all these substrates in the presence of similar tissue, similarly collected.

Critical examination of the method for estimating lactose which these authors used (Scott & West, 1936) suggests that, although it is applicable to simple mixtures of sugars, e.g. glucose, galactose and lactose, for which purpose it was, apparently, originally designed, it must be used with only the greatest caution in any work involving biological systems of greater complexity, where contaminants may be present which can give rise to reducing substances on hydrolysis. The best illustration of the pitfalls of this method when applied to the problem of lactose synthesis by the mammary gland arises out of our demonstration of the presence of amylolytic activity in all the glands, cow or guinea pig, with which we have dealt. It would be irrelevant at this point to discuss whether or no this activity arises from a true mammary amylase, or is due to enzymes from blood or tissue fluids adsorbed on the

tissue; but at least the activity persists after extensive washing of the tissue slices, and the fact remains that dextrins, and probably maltose too, are formed in these incubations when glycogen is the substrate; these substances, by giving rise to glucose on hydrolysis would lead to completely false estimates of lactose synthesis from glycogen by the Scott & West (1936) method.

Knodt & Petersen's (1945) further claim that glucose and maltose also promote lactose synthesis in the cow gland cannot be explained in identical terms, for it is unlikely that in these cases there could have been any appreciable formation of dextrins in their incubated solutions; but an instance of the inherent dangers of the Scott & West (1936) method in these experiments, even when simple sugars are used as substrates, is given by some experiments we ourselves carried out on cow glands with glucose as the substrate, using the Scott & West (1936) method for estimating lactose. Contrasting with the small increase in reducing power given by our normal, direct reading of the solution after fermentation (mean value as 'lactose' 0·4 mg.), and the slightly negative methylamine values, we obtained an apparent lactose synthesis by the Scott & West method (modified to the extent of using N-hydrochloric instead of N-sulphuric acid for the hydrolysis, neutralizing with N-sodium hydroxide and estimating reducing power by Somogyi's (1945) method) of 6·2 and 5·5 mg. per g. dry weight of tissue in a 1 hr. incubation period. Knodt & Petersen's (1945) corresponding mean value is 2·2 (13·3 for a 6 hr. incubation period), a figure obtained by multiplying their wet weight value by 6·5. This factor for the conversion of wet to dry weights was derived from our own measurements on cow mammary tissue, and is presumably applicable here with only small error. Although it is unlikely that dextrins were responsible for these high readings, it is possible to suggest other substances, of which glucose-1-phosphate is the most obvious, which could interfere with the Scott & West method in very much the same, misleading, way.

While, therefore, the evidence from arterio-venous sampling *in vivo* abundantly suggests that glucose is a precursor of lactose in the cow udder, we feel that the synthesis of lactose by cow tissue has not yet been satisfactorily demonstrated *in vitro*. Since in this work, and in that of Grant (1935), there has been no difficulty in proving synthesis of lactose *in vitro* by the guinea pig gland, it is pertinent to inquire why cow glands should prove to be so refractory. Three suggestions may be put forward at this time. First, we may suppose that the glands used, although milky in appearance were, in fact, regressive and had lost the power of synthetic activity; this supposition remains untested. Secondly, it could be claimed that since no special precautions were taken when collecting the udders from the slaughterhouse, the tissues may have undergone some permanent damage in the 1·0–1·5 hr. between slaughter and the start of the experiment, sufficient to prevent lactose synthesis. We have attempted to check this possibility by comparing the lactose synthesis from glucose given by slices of the same guinea pig gland, cut and incubated (*a*) immediately after killing, and (*b*) after the gland had remained at room temperature for 1·5 hr., just moistened with a few drops of Krebs Ringer phosphate solution. The results showed no appreciable change in the ability of the gland to form lactose, the synthesis by reduction being (*a*) 6·5 mg., (*b*) 4·6 mg. and by the methylamine method (*a*) 4·5 mg., (*b*) 4·3 mg. We might, therefore, tentatively exclude this possibility of a deterioration of active udder tissue in the interim period between killing and the start of an experiment. There remains the third possibility that glucose alone is insufficient as a precursor for lactose synthesis by the cow gland, and that this gland differs from that of the guinea pig in requiring an additional substrate or substrates. Graham, Jones & Kay (1936), and others, have drawn attention to the very high rate of blood flow through the mammary gland which would be required to satisfy the needs for lactose synthesis if blood glucose were the only precursor in the cow, and the suggestion derives further support from the recent work of Folley & French (1948) on comparative differences in the fat metabolism of the mammary glands of ruminants and small laboratory mammals. Their demonstration of the ability of ruminants to utilize acetate in fat synthesis, and their later observation that the trivial acetate uptake by the rabbit mammary gland could be greatly increased by simultaneous glucose administration (Folley & French, 1949) are results which might well have a counterpart in the field of comparative carbohydrate metabolism.

Our results with guinea pig glands agree with those of Grant (1935) in establishing glucose as a precursor for lactose in this species, and extend his observations to include glycogen as a second source of the disaccharide. The fact that glucose customarily gave the larger lactose synthesis makes it less likely that glycogen is an obligatory intermediary in lactose formation (Knodt & Petersen, 1946), and implies rather that its role is one of a minor carbohydrate reserve. Further, since maltose gives rise to no lactose when used as the substrate for mammary tissue, it is most improbable that the metabolic pathway from glycogen to lactose will involve any amylolytic breakdown; we may therefore postulate at this stage the likelihood of a phosphorolytic scheme of glycogenolysis in which either glucose-1-phosphate or glucose-6-phosphate may prove to be a common intermediary on both the glycogen and glucose pathways to lactose formation.

SUMMARY

1. The synthesis of lactose from glucose and glycogen by tissue slices of guinea pig mammary gland has been demonstrated chromatographically, and an attempt to evaluate the synthesis quantitatively has been made by a consideration of the results of two entirely unrelated chemical estimations.

2. No lactose synthesis from glucose or glycogen by tissue slices of cow mammary gland could be demonstrated; a critical assessment of previous claims leads to the conclusion that no *in vitro* demonstration of lactose synthesis by the ruminant gland has yet been made.

3. Maltose (cow and guinea pig glands) and lactate (guinea pig gland) proved ineffective as substrates for lactose synthesis.

We wish to express our thanks to Prof. D. C. Harrison for his interest in the progress of this work, and to Dr Q. H. Gibson for helpful criticism of the manuscript. We are indebted to Messrs Caffrey, Belfast, for regular supplies of brewer's yeast, and to Messrs Gillette Industries Ltd. for a generous gift of special razor blades.

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The Valences of Protein Ions from Electrophoretic and Membrane Potential Measurements

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The moving boundary method of electrophoresis, developed by Tiselius (1930, 1937), Philpot (1938), Longsworth (1939), and Svensson (1939, 1940), has many applications, including the small-scale preparative separation of protein mixtures. The purpose of the present studies was ultimately to obtain information likely to be of use in the electrophoretic separation of interesting fractions from pathological human sera.

Convection, due to various causes, is a major difficulty in electrophoresis. The passage of a boundary through the bottom channel of the U-tube due to any cause sets up gravitationally unstable conditions and hence convection, and this is true of the so-called δ-boundary, the origin of which has been discussed by Longsworth & MacInnes (1940).

The separation of pure samples of the components *A* and *B* of a binary protein mixture is illustrated in Fig. 1. If diffusion is neglected, the best possible separation is shown in Fig. 1*a*. If the δ-boundary is eliminated, or reduced to such an extent that its passage through the bottom channel of the U-tube results in negligible convections, the separation theoretically possible is improved to that shown in Fig. 1*b* (Svensson, 1948).

The practical elimination of the δ-boundary may be accomplished either by dilution of the dialysed protein solution (Longsworth & MacInnes, 1940), or by the use of a supernatant buffer solution rather more concentrated than that used for the dialysis (Wiedemann, 1947). There are some objections to these procedures (Pedersen, 1948). Thus, for analy-

tical work it is better to reduce anomalies by suitable choice of protein concentration and buffer (Longsworth, 1942; Svensson, 1946); however, as mentioned by Longsworth (1947), the above modifications may be useful in preparative work.

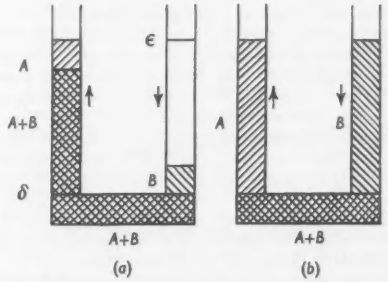


Fig. 1. Theoretical separation of mixture $A+B$ by electrophoresis (*a*) under ordinary conditions, (*b*) when the δ-boundary has been eliminated.

According to the classical theory of Kohlrausch (1897) the development of δ- (and ε-) boundaries is due to the different values of the 'beharrliche Funktion', or regulating function, for the dialysed protein solution and the buffer against which it has been dialysed. This buffer will be referred to as the dialysate, in conformity with Adair & Adair (1934). If we know the mobilities of all the ions present in the protein solution and their concentrations in terms of electrochemical equivalents, we can calculate the regulating function, $\Sigma c_1/u_1$ in the notation below, and hence the dilution factor, or the excess concentration of buffer in the supernatant, required to eliminate the δ- and ε-boundaries. If all the above information is available, it is probable that the components are already so well characterized as to render of little interest their isolation by the method under discussion. It was therefore decided to work with a comparatively pure protein preparation, and by making arbitrary modifications in the strength of the supernatant buffer, to estimate the least supernatant concentration at which the δ- and ε-boundaries were completely eliminated. The regulating functions of the protein solution and the supernatant should then be equal. This information, together with the electroneutrality relation and the equation of the original Donnan equilibrium set up on dialysis, suffices for the estimation of the protein concentration in terms of electrochemical equivalents and hence the valence (Longsworth, 1947).

Measurements were made on the δ-, rather than on the ε-boundary, as the former is larger and measurable more accurately. Although a single observation of the ε-boundary would suffice for deducing the regulating function of the protein solution (Longsworth, 1947), it was anticipated that the error might be less if a series of measurements was made, as in this work.

A completely homogeneous protein preparation would have been desirable for these studies, but such a material is difficult to prepare (Pirie, 1940; Alberty, Anderson & Williams, 1948). An albumin was selected because the relatively high mobilities can be measured accurately, and there is a good separation from the δ-boundary. The albumin used was horse serum albumin A (Kekwick, 1938), provided by Dr R. A. Kekwick.

Valences determined by the above method were compared with those calculated from the electrophoretic mobilities observed under similar conditions. In those cases where the effects were measurable within reasonable limits, membrane potentials were determined, yielding a third independent set of values for the valences. Comparative studies of protein-ion valences obtained by different methods have been relatively few in number (Tiselius & Svensson, 1940; Adair & Adair, 1940; Longsworth, 1941; Abramson, Moyer & Gorin, 1942; Creeth & Jordan, 1949), but great interest attaches to the results in view of the assumptions and approximations made in the different methods.

THEORETICAL

(*a*) *Moving boundary method.* If c = concentration in electrochemical equiv./l., taken with the appropriate sign, suffixes *A*, *R*, *Pr* refer respectively to buffer cation, buffer anion, and protein ion, suffixes 1, 2, 3 refer respectively to conditions inside the dialysis bag, in the dialysate and in the supernatant buffer when the δ- and ε-boundaries are eliminated, then the electroneutrality condition gives

$$c_{A,1}+c_{R,1}+c_{Pr,1}=0. \quad (1)$$

If activity coefficients for the same ion on both sides of the dialysis membrane may be considered equal, from the Donnan equilibrium,

$$\left(\frac{c_{A,1}}{c_{A,2}}\right)^{1/z_A}=\left(\frac{c_{R,1}}{c_{R,2}}\right)^{1/z_R}, \quad (2)$$

where z_R, z_A are the valences of the anion and cation respectively, taken with the appropriate sign. The Kohlrausch (1897) regulating function gives

$$\frac{c_{A,1}}{u_A}+\frac{c_{R,1}}{u_R}+\frac{c_{Pr,1}}{u_{Pr}}=c_{A,3}\left(\frac{1}{u_A}-\frac{1}{u_R}\right), \quad (3)$$

where u refers to mobility, including the requisite sign. For the Na acetate solutions $z_A=1$, $z_R=-1$ and for the Na phosphate $z_A=1$, $z_R=-2$. In these cases the best method of solution is by elimination of $c_{R,1}$ and $c_{Pr,1}$ from equations (1) to (3), yielding respectively

$$c_{A,1}^2\left(\frac{1}{u_A}-\frac{1}{u_{Pr}}\right)+c_{A,1}c_{A,3}\left(\frac{1}{u_R}-\frac{1}{u_A}\right)+c_{A,2}c_{R,2}\left(\frac{1}{u_R}-\frac{1}{u_{Pr}}\right)=0, \quad (4)$$

$$c_{A,1}^3\left(\frac{1}{u_A}-\frac{1}{u_{Pr}}\right)+c_{A,1}^2c_{A,3}\left(\frac{1}{u_R}-\frac{1}{u_A}\right)+c_{A,2}^2c_{R,2}\left(\frac{1}{u_R}-\frac{1}{u_{Pr}}\right)=0. \quad (5)$$

These equations contain the single unknown quantity $c_{A,1}$, which may be evaluated by standard algebraical procedures since the value of $c_{A,2}$ is obtained experimentally, as described below. The method of Newton or Horner enables the cubic equation to be solved to any degree of approximation. Substitution of the value of $c_{A,1}$ in the appropriate equation (2) gives the corresponding value of $c_{R,1}$. Then, $c_{Pr,1}$ follows easily from equation (1). It has been found that solution in this way is preferable to the elimination of $c_{A,1}$ and $c_{R,1}$ from equations (1) to (3), which results in clumsy expressions for the quadratic and cubic equations in $c_{Pr,1}$. The protein ion valence, z_{Pr}, can be deduced from $c_{Pr,1}$, provided the stoicheiometric concentration of protein is measured and the molecular weight known.

(*b*) *Mobility method.* The method of Abramson, Gorin & Moyer (1939) and Abramson *et al.* (1942) was used to calculate valences from the relation

$$z = \frac{6\pi\eta r}{f(\kappa r)} \frac{(1+\kappa r+\kappa r_i)}{(1+\kappa r_i)} u \times \frac{300}{4{\cdot}80\times10^{-10}},$$

where r = radius of protein ion (assumed spherical); r_i = average radius of ions of ionic atmosphere; κ = the Debye-Hückel ionic strength function; $f(\kappa r)$ = Henry's function; η = viscosity of solvent.

In this relation, a modification of the formula of Henry (1931), derived on the basis of the theory of Debye & Hückel (1923), several assumptions and limitations are implicit; these are discussed in detail by Abramson *et al.* (1939, 1942). One such limitation is that the equation can be applied only in the case of a spherical ion, but the authors of the method evolved a means of applying the relation to cylindrical particles, by expressing the mobilities of such particles in terms of those of spheres of the same molecular volume.

(*c*) *Membrane potentials.* The measured potential difference between the protein solution and the dialysate is given by the relation

$$E = -\frac{RT}{z_A F}\ln\frac{c_{A,1}}{c_{A,2}} = -\frac{RT}{z_R F}\ln\frac{c_{R,1}}{c_{R,2}} \qquad (6)$$

where R, T, F have their usual significance. This equation has the same implications regarding activity coefficients as has equation (2).

A very full discussion of the errors inherent in the membrane potential method has been given by Adair & Adair (1934). Probably the chief sources of error are the liquid junction potentials, but these can be considerably reduced by use of saturated KCl solution in the electrode elements. Adair & Adair (1934) have shown that, under the conditions of these experiments, the remarks above about activity coefficients are sufficiently close to the truth. The possibility of alterations in the solutions during equilibration has been minimized by working near 0° in stoppered Pyrex vessels, and by avoiding the use of excessively dilute buffers. Owing to the low protein concentrations used the volume correction of Adair & Adair (1934) has been dispensed with.

The protein-ion valence can be calculated with the help of equations (1) and (6).

EXPERIMENTAL

Electrophoresis experiments. These were carried out in the Tiselius (1937) apparatus at +0·5°. The protein solutions were dialysed in cellophan bags for 4–5 days against the appropriate buffer solutions, the dialysates being changed twice daily. Three sets of measurements were carried out at different protein concentrations (0·5 – 1·5%) after dialysis against Na acetate-acetic acid buffer, ionic strength 0·02, pH 3·82. Five other sets were carried out at approximately 1·4% protein concentration dialysed against disodium phosphate solutions of various ionic strengths. The more dilute phosphate solutions differed somewhat in pH from the more concentrated (Table 1), indicating the presence of small amounts of monovalent phosphate ion. The latter was ignored in the calculations. Potential gradients used in the U-tube ranged from 3·0 to 9·5 V./cm. according to the buffer. Measurements of the area of the δ-boundary were carried out by the Svensson (1939, 1940, 1946) technique, as this gives more consistent results with small refractive index differences measured at large inclinations of the diagonal slit to the vertical, when the base line may deviate appreciably from linear (Svensson, 1946). Mobility measurements were made in solutions with a fairly low protein to buffer ratio (Longsworth, 1942; Svensson, 1946), approximately 1% protein being used in buffer of ionic strength 0·2, and proportionally less protein at lower ionic strengths. Even so the ascending boundary moved about 2% faster than the descending. Consequently, mobilities were measured in the descending limb, the conductivity of the protein solution being used for computation (Svensson, 1946). Corrections for volume changes at the electrodes were considered to be negligible in these experiments (Longsworth, 1942, 1943). The positions of the boundaries were identified with those of the median ordinates (Longsworth, 1941, 1943), estimated from patterns traced on millimetre graph paper with an enlargement factor of five. From the mercury vapour lamp light source monochromatic light (λ = 546 mμ.) was isolated by means of a filter. The patterns were recorded on Ilford thin film half-tone panchromatic plates. Conductivity measurements were made in a Washburn-type conductivity cell immersed in the electrophoresis bath at +0·5°.

Membrane potentials were measured at +0·5° by a technique closely similar to that of Adair & Adair (1934). Two modifications were made. Good quality cellophan tubing was tied at one end and supported by sliding it over the end of a short test tube in which several large holes had been blown. The upper end of the cellophan was held tightly to the tube by a stout rubber band. The levels of both protein solution and dialysate were kept below the level of the rubber band. No necessity was felt to work at the equilibrium osmotic pressures of the solutions in view of the conclusions of Northrop & Kunitz (1926) and Adair & Adair (1934). Dialysis was able to proceed freely under the above conditions, and the membranes were protected against damage from the electrode capillary tubes during measurements. Instead of calomel electrodes Ag-AgCl in saturated KCl were used. These were prepared according to MacInnes & Beattie (1920). Polarization was negligible.

Boundaries were formed in the electrode capillary U-tubes by the method of Maclagan (1929), and readings taken 5–10 min. later. If contamination of the solutions by KCl occurred at this stage, dialysis had to be repeated. The potential difference between two electrodes was measured, then the potential when they were immersed in protein solution and dialysate respectively, and finally the electrodes were checked against each other. This procedure was repeated, but the electrode previously dipped into the dialysate was this time used in the protein solution. The membrane potentials, corrected for the electrode differences, were then averaged. Owing to liquid-junction potentials it was difficult to obtain reproducibility better than 0·1 mV. At the

protein concentrations used only measurements in ionic strengths of 0·02 and below were sufficiently large to yield results of any quantitative significance.

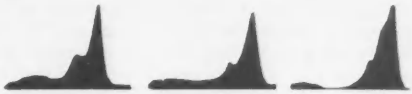
Nitrogen estimations were carried out by the method of Ma & Zuazaga (1942), but Na selenate replaced selenium in the digestion catalyst, and digestion was for at least 4 hr. The conversion factor of 6·97 was used for this protein (Kekwick, 1938).

pH *measurements* were made at 20° by means of a Cambridge pH meter with glass electrode. 0·05 M-Potassium hydrogen phthalate (pH 3·97) and 0·05 M-borax (pH 9·18) were taken as standards.

RESULTS

Patterns obtained by electrophoresis of samples of the horse serum albumin A are shown in Fig. 2. There are indications of the presence of a small amount of globulin impurity; furthermore, the albumin peak in Fig. 2 (*a*) and (*c*) shows evidence of being composite in accordance with the observations of Luetscher (1939).

series are not necessarily directly comparable. The results of experiments in acetate buffer are shown in Fig. 5.



(*a*) (*b*) (*c*)

Fig. 2. Electrophoretic patterns given by preparation of horse serum albumin A. Migration is from left to right. (*a*) Sodium acetate-acetic acid, $I=0{\cdot}2$, pH 3·8. Protein 1·2%. After 369 min. at 3·5 V./cm. (*b*) Sodium phosphate, $I=0{\cdot}05$; sodium chloride, $I=0{\cdot}15$; pH 8·0. Protein 0·9%. After 349 min. at 2·3 V./cm. (*c*) Sodium phosphate, $I=0{\cdot}2$, pH 8·8. Protein 0·9%. After 216 min. at 2·8 V./cm.

From the intercept on the abscissae of the curves in Figs. 4 and 5 the value of $c_{A,3}$ can be obtained directly for substitution in equations (4) and (5).

(*a*) (*b*) (*c*) (*d*) (*e*)

Fig. 3. Philpot-Svensson curves showing the reduction in size of the δ-boundary as the excess concentration of buffer in the supernatant is increased from (*a*) 0%, through (*b*) 15%, (*c*) 25%, (*d*) 35%, to (*e*) 40%. Results obtained with a solution of 1·5% horse serum albumin A; dialysate disodium phosphate, $I=0{\cdot}02$, pH 8·2.

A typical series of patterns of the δ-boundaries, obtained in 0·02 ionic strength phosphate buffer, is shown in Fig. 3. The results of all the experiments in phosphate buffers are shown in Fig. 4. Although the diagonal slit was kept at a set inclination throughout a particular series, the areas obtained in different

Convection is very liable to occur in solutions where the protein to buffer ratio is too high, whether or not the ordinary dialysate is employed as supernatant. This is especially true of the region just below the descending albumin peak. In confirmation of the warning of Pedersen (1948), there was an in-

Table 1. *Horse serum albumin valences calculated from moving boundary studies in phosphate buffers*

Dialysate: Ionic strength, $I=\frac{3}{2}c_{A,2}$	Dialysate: pH	Excess buffer concentration to eliminate δ-boundary (%) $=100\,(c_{A,3}/c_{A,2}-1)$	Total N (mg./ml.)	Protein concentration (electrochemical equiv./l.) $=c_{Pr,1}$	Valence $=z_{Pr}$
0·2	8·83	8·0	1·99	−0·00655	−33·2
0·1	8·81	12·4	1·99	−0·00618	−31·3
0·05	8·63	19·5	1·95	−0·00556	−28·8
0·02	8·22	38·4	2·15	−0·00508	−23·9
0·01	8·05	59·6	1·68	−0·00337	−20·3

Table 2. *Horse serum albumin valences calculated from moving boundary studies in acetate buffers*

Dialysate: Ionic strength, $I=c_{A,2}$	Dialysate: pH	Excess buffer concentration to eliminate δ-boundary (%) $=100\,(c_{A,3}/c_{A,2}-1)$	Total N (mg./ml.)	Protein concentration (electrochemical equiv./l.) $=c_{Pr,1}$	Valence $=z_{Pr}$
0·02	3·82	20·2	0·774	0·00267	+34·8
0·02	3·82	32·7	1·39	0·00429	+31·1
0·02	3·82	76·5	2·69	0·00982	+36·8

creased tendency to convection just below the δ-boundary as the limiting concentration of buffer was reached in the supernatant. It is, of course, obvious that convections are inevitable in this region should this limiting concentration be exceeded. The conditions employed in the mobility determinations ensured that the solutions were free from detectable convection.

$$u_{Na}^{+} = 23{\cdot}5 \times 10^{-5} \text{ cm.}^2\text{V.}^{-1}\text{sec.}^{-1}$$
$$u_{HPO_4}^{--} = -21{\cdot}9 \times 10^{-5} \text{ cm.}^2\text{V.}^{-1}\text{sec.}^{-1}$$

after Hoch (1948) and

$$u_{CH_3COO^-} = -18{\cdot}5 \times 10^{-5} \text{ cm.}^2\text{V.}^{-1}\text{sec.}^{-1},$$

based on the relative mobility of acetate ion given by Longsworth (1947). Protein-ion mobilities used were the measured values shown in Table 3.

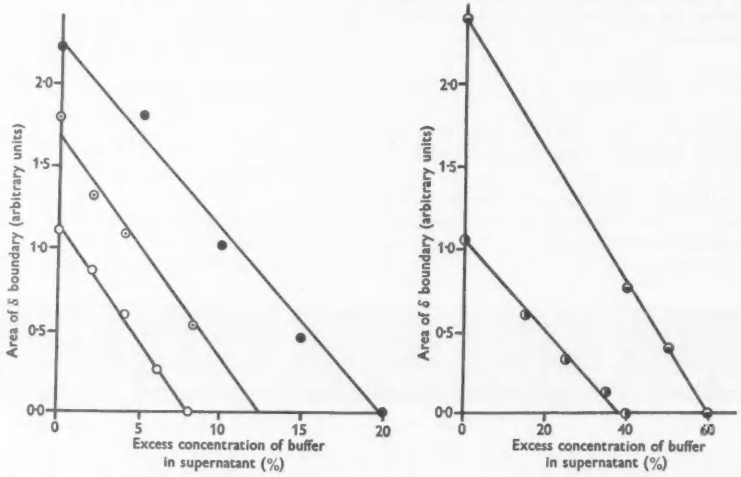

Area of δ boundary (arbitrary units)
Excess concentration of buffer in supernatant (%)
Area of δ boundary (arbitrary units)
Excess concentration of buffer in supernatant (%)

Fig. 4. Elimination of the δ-boundary in the phosphate buffers of Tables 1 and 3. Points correspond to ionic strengths as follows: ○, 0·2; ⊙, 0·1; ●, 0·05; ◑, 0·02; ◒, 0·01.

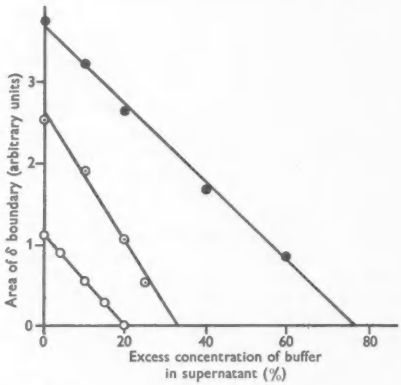


Fig. 5. Elimination of the δ-boundary. Dialysate acetate buffer, $I = 0{\cdot}02$, pH 3·8. Points correspond to albumin concentrations as follows: ○, 0·5%; ⊙, 1·0%; ●, 1·8%.

Tables 1 and 2 show the values of the valences calculated from the moving-boundary studies by the method discussed above. In these calculations the following mobility values were used:

Tables 3 and 4 show, respectively, the valences calculated from the mobility data and from the membrane potentials. In the calculations from mobility data the physical constants of the protein were assumed equal to those used by Abramson *et al.* (1942) for horse serum albumin B, on the basis of the sedimentation and diffusion measurements of Kekwick (1938). Two sets of valences are recorded, based on the extreme molecular models of a hydrated sphere and an unhydrated cylinder of axial ratio 5/1. A value of 2·5 A. was retained for r_i in phosphate buffers. This was considered justified on the grounds that: (*a*) sodium ions will predominate in the ionic atmosphere close to the negatively charged protein ion; (*b*) the phosphate ion will probably not differ greatly in size from this value; and (*c*) this factor affects the results relatively little except at the higher ionic strengths.

DISCUSSION

The most direct method of determining protein-ion valences would appear to be from observations of electrophoretic mobility, but considerable difficulties arise in the theory (Abramson *et al.* 1942). In

Table 3. *Horse serum albumin valences calculated from mobility measurements*

Dialysate: Buffer	Dialysate: Ionic strength I	Dialysate: pH	Mobility, u_{Pr} ($cm.^2 V.^{-1} sec.^{-1} \times 10^{-5}$)	Valence (for hydrated sphere) z_{Pr}	Valence (for an unhydrated rod of axial ratio 5/1) z_{Pr}
Phosphate	0·2	8·83	− 6·3	−18·5	−20·6
	0·1	8·81	− 7·5	−18·5	−20·1
	0·05	8·63	− 8·5	−17·8	−19·1
	0·02	8·22	− 9·9	−16·5	−17·6
	0·01	8·05	−11·8	−16·7	−17·5
Acetate	0·02	3·82	+ 7·2	+12·0	+12·8

Table 4. *Horse serum albumin valences calculated from membrane potential measurements*

Dialysate: Buffer	Dialysate: Ionic strength (I)	Dialysate: pH	Membrane potential (mV.)	Total N (mg./ml.)	Protein concentration (electrochemical equiv./l.) $c_{Pr,1}$	Valence, z_{Pr}
Phosphate	0·02	8·22	−2·31	1·92	−0·00374	−19·5
	0·01	8·05	−3·70	1·79	−0·00293	−16·4
Acetate	0·02	3·86	+2·55	3·30	+0·00433	+13·2
	0·02	3·85	+1·89	2·70	+0·00320	+12·0
	0·02	3·86	+1·58	2·13	+0·00264	+12·5
	0·02	3·85	+1·22	1·52	+0·00206	+13·7

addition, there are specific buffer-ion effects (Svensson, 1946). A new theoretical approach has recently been made by Booth (1948). Other methods depend mainly on the estimation of inorganic ions and application of the electroneutrality equation. Chemical analysis, either directly (Svensson, 1946) or by isotope dilution (Velick, 1949), gives the total of free and combined material. Titration data may be lacking in accuracy, unless suitable corrections are made for the combination of the protein ions with ions other than hydrogen or hydroxyl, or with undissociated molecules (Tiselius & Svensson, 1940; Abramson *et al.* 1942). Membrane potential measurements may involve the errors already discussed. The first comparative studies (Tiselius & Svensson, 1940; Adair & Adair, 1940) did not take into account several factors, so that the apparent agreement between mobility and membrane potential values depended to some extent on compensating errors (Longsworth, 1941; Abramson *et al.* 1942). The recent results of Creeth & Jordan (1949) show excellent agreement at low ionic strengths in the case of the highly asymmetric particles of nucleic acids.

The valences deduced from δ-boundary measurements assume that the charge operative in membrane equilibria is identical with that on the ion migrating in an electric field. Evidence is accumulating in support of this (Svensson, 1946). Thus valences calculated from membrane potentials and from mobility measurements are in reasonably good agreement. In addition to the above assumption of identity of charge, there is the requirement of the Kohlrausch (1897) theory that the mobility of a given ion should be independent of its position in the U-tube. In practice there may be appreciable alterations of pH across the boundaries (Longsworth, 1947), and the variation of protein ion mobility with pH is usually quite large. Under some conditions in the present experiments the buffering capacity of the salts present is not as great as would be desirable. In some cases this is due to the endeavours to obtain measurements under the same conditions as used for membrane potentials. The disodium phosphate solutions of pH 8·8 also have poor buffering capacity; these solutions were selected to simplify the algebra involved. In calculation no account has been taken of possible contributions of hydrogen ions, etc., to the conductivity, or various equilibria. In order partly to suppress changes of pH at boundaries, the acetic acid/sodium acetate ratio in the supernatant was kept constant, in contrast to the procedure of Longsworth (1947), who diluted his protein solution with acetic acid solution.

The present work shows quite good correspondence between the valences determined from membrane potentials and those from mobilities, particularly where the latter are calculated on the basis of a cylindrical model which approximates to the most probable form of the horse serum albumin molecule (Oncley, 1941). It should be noted that the protein solutions used for mobility measurements were at practically the same pH values as the corresponding dialysates, whereas the relatively high protein concentrations necessary in the membrane potential measurements involved a change of pH across the membrane amounting to approximately 0·07 unit (calc.) in some cases.

The valences calculated from mobility measurements in phosphate solutions are subject to specific influences (Longsworth, 1941); but it was desired to work under conditions approximating to those liable to be encountered in the course of electrophoretic separations and phosphate buffers are commonly used for such work, particularly with serum proteins. The agreement with the membrane potential results is perhaps slightly better than might therefore be expected.

The valences calculated from the δ-boundary measurements are considerably larger than those deduced by the other methods. The best agreement is in phosphate of low ionic strength, where the anomalies might be expected to be larger. The apparent variation of charge with ionic strength in phosphate buffers is partly due to the differences in pH. The only data available for comparison are those of Longsworth (1947), who obtained from ε-boundary measurements a value of 10·5 for the valence of ovalbumin in acetate buffer of pH 3·92 and ionic strength 0·1. Although this corresponds well with titration data, Abramson *et al.* (1942) have shown that correction of the titration figures reduces the value to approximately 5·0, in accordance with mobility calculations. Thus the results of Longsworth also are rather higher than would be expected. Since neither Longsworth's ovalbumin, nor the present horse serum albumin, is a homogeneous material the deviations could be due partly to this cause; but, no doubt, must be attributed mainly to the inadequacy of the Kohlrausch (1897) theory for the present systems. Recent theoretical advances (Dole, 1945; Svensson, 1946) are not easy to apply in a general case, although Hoch (1948) has obtained promising results based on the Svensson (1946) theory, when certain assumptions are made regarding protein interactions. Further improvements in correlating results of the various methods of determining the valences of protein ions may be expected when the new theories are applied in studies conducted on the relatively pure proteins which are becoming available through the researches into fractionation methods such as those of Cohn, Hughes & Weare (1947).

The conclusion to be drawn from the results of the δ-boundary experiments is that, at present, it is not possible in eliminating the δ-boundary for preparative work to improve on the following semi-empirical method. From a preliminary run and measurement of the δ- or ε-boundary the buffer concentration needed in the supernatant is calculated. A second run using this strength buffer will generally still result in δ- and ε-boundaries, smaller in size. A second, and if necessary further, approximation results in the required elimination. Under these circumstances the ideal separation illustrated in Fig. 1 (*b*) may be almost attained in the ascending limb, where there is a greater sharpening effect than usual, whilst in the descending limb the modified conditions favour more diffuse boundaries whereby the increased separation is partly, or even wholly, offset.

SUMMARY

1. Measurements have been made in acetate and phosphate buffers of the variations in size of the δ-boundary formed during the electrophoresis of horse serum albumin A with modified concentrations of buffer salts in the supernatant fluid.

2. The valences of the protein ions calculated from these observations have been compared with those deduced from mobility and membrane potential measurements.

3. The last two methods compare very favourably, but the first one gives rather higher values. The reasons for this are discussed.

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The Selective Hydrolysis of Amylopectin and Amylose by β-Amylase

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The common starches are now generally recognized as consisting of a branched component (amylopectin) and a linear one (amylose), hydrolysable by β-amylase which attacks the α-glucosidic chains at their non-reducing ends to yield about 50 and 100% maltose respectively. Potato amylopectin has one such chain end for every 24–26 glucosidic groups present, whereas potato amylose has one to every 200–500 or more.

An investigation has been undertaken of the relative rates of disappearance of these two components of starch during hydrolysis by β-amylase. The proportion of amylose and amylopectin remaining in a partly hydrolysed starch paste can be calculated from the absorption spectra of its iodine colour under standard conditions. If amylopectin is preferentially hydrolysed amylose will accumulate and the iodine colour will be relatively bluer.

MATERIALS AND METHODS

Substrates. Three commercial samples of potato starch, one each of wheat, rice, a soluble starch (A.R.) and a Lintner soluble starch prepared in our laboratory from potato starch were used. Amylose was prepared from potato starch by the thymol precipitation method of Bourne, Donnison, Haworth & Peat (1948), and amylopectin by *cyclo*hexanol and thymol precipitation as described by Hopkins & Jelinek (1948). In some cases the amylopectin was preserved in solution, sterile.

Starch and amylopectin were made into a cream with cold water, poured into boiling water and boiled with continuous mechanical stirring for 15 min. With native starches, 0·05 g. of NaCl/100 ml. was added 10 min. before the end of boiling. Amylose was preserved under butanol and dissolved in hot water freshly for use, the butanol being driven off at about 80–90°.

Preparation of amylopectin. The purest preparation of amylopectin made in these laboratories was obtained by precipitation of potato starch paste with *cyclo*hexanol followed 3 days later by thymol (Hopkins & Jelinek, 1948). It had been stored for 2 years and was difficult to get into solution unless finely ground, and all the precautions required in the preparation of starch paste were employed. A white flocculum was thrown out, apparently retrograded amylose, which was separated and the solution centrifuged clear. There were indications that unless it was well dispersed it hydrolysed only half as rapidly as other specimens of amylopectin. The data on which Fig. 1 was constructed were obtained with this sample.

Preparation of amylose and amylopectin for determination of Michaelis constants. Potato starch was stirred with water at 63° for 15 min., the clear portion separated and the amylose prepared from it by thymol precipitation (amylose I, yield 4·6–6·2%). The starch residue was dispersed by boiling for 30 min., centrifuged to remove small amounts of residues, and the residual amylose (II) precipitated by thymol, leaving amylopectin II. The amylose preparations were purified and stored as described by Hopkins & Jelinek (1948). The amylopectins were kept in sterile solution, amylopectin II for 3 months and amylopectin III (prepared in the same way as II) for 6 days. (Amylopectin I derived from the 63° infusion (yield 0·8–1·1%), will be referred to later.)

Amylose I corresponds to the β-amylose of Baldwin (1930) and the amylose of Meyer, Bernfeld & Wolff (1940), but, having been precipitated with thymol, it should be free from amylopectin. In Fig. 2 it is described as 'Baldwin amylose'.

Enzyme preparations. Both β-soya and barley amylase were prepared as described by Hopkins, Jelinek & Harrison (1948).

Reaction mixtures. Conditions of pH, temperature, etc., are given in the appropriate tables or in the text. In view of the effects of salts on spectrometric measurements of iodine coloration, the concentration of buffer solutions was kept down to a 1% addition of 0·1 M-acetate (for pH 4·6) and citrate-phosphate for pH 5·8 and 7.

Progress of hydrolysis was followed and Michaelis constants were determined as described by Hopkins *et al.* (1948).

Iodine absorption spectra were determined with appropriate solutions in a 1 cm. cell in a Hilger Spekker absorptiometer at 20° using, in combination with the heat filter (H 503), the Ilford filters 608 (6800–7000 A. transmission), 607 (6000 A.), 603 (4900 A.) and 602 (4700 A.). These solutions contained 10 mg. of I_2 + 100 mg. of KI/100 ml. together with 5 mg. of starch or amylopectin or its equivalent from a reaction mixture undergoing hydrolysis, but in the case of amylose only 1 mg. was present. In all cases the solution was kept at 20° for 2 hr. to enable the full colour to develop, and allowance was made for the colour due to I_2 alone by making the reading against a corresponding I_2 solution.

RESULTS

Calibration of Spekker absorptiometer for the measurement of amylose, amylopectin and α-amylodextrin. Amylose (0–1·2 mg./100 ml.), amylopectin (0–6 mg.) and α-amylodextrin (0–8 mg.) were found to give linear relationships between carbohydrate concentration and absorptiometer reading for each colour filter. One mg. of amylose/100 ml. gave readings 0·390, 0·350, 0·145, 0·124 for the 608, 607, 603 and 602 filters respectively; those for amylopectin can be read from Fig. 1.

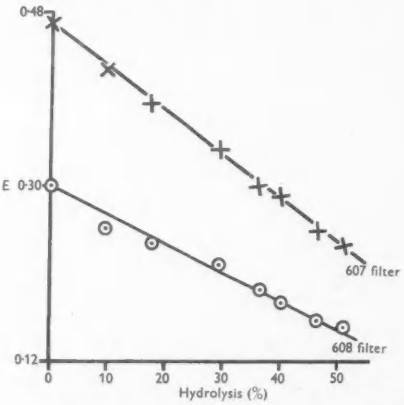

Fig. 1. Iodine coloration of amylopectin during hydrolysis by β-amylase. Ordinates. Extinctions (Spekker absorptiometer readings) 5 mg. of amylopectin (or its equivalent during hydrolysis) per 100 ml.; ×—×—×, Ilford filter no. 607; ⊙—⊙—⊙, Ilford filter no. 608.

The readings for amylose and amylopectin bore an additive relationship over all ranges up to and including 0·8 mg. of amylose and 5 mg. of amylopectin per 100 ml. It was thus possible to calculate from such readings the quantities of amylose and amylopectin in mixtures of the two. It remained to determine how their respective iodine colour values change during the progress of hydrolysis by β-amylase. Such volume of reaction mixture as contained 5 mg. of amylopectin at the start was withdrawn at intervals for readings in the absorptiometer. The latter, plotted against the percentage hydrolysis, gave straight lines to 51 % (Fig. 1). From these data, the corresponding figures for 5 mg. of the residual polysaccharide per 100 ml. were calculated, thus bringing all the values to the same basis. These results were approximately constant for any one filter. The same is true of amylose, readings, calculated to the basis of 1 mg. of residual polysaccharide per 100 ml., remaining constant until the hydrolysis is well advanced.

From all these observations it was concluded that the relative rates of disappearance of amylose and amylopectin could be calculated from the absorptiometer readings and percentage hydrolysis, provided the process was confined to the hydrolysis of about 50 % of the amylopectin. The 608 and 607 filters should be used, as by far the greatest difference between amylose and amylopectin is observed with them.

Certain precautionary tests were made. It was desirable to dilute amylose as far as possible before adding the iodine, otherwise the amylose-iodine complex tended to flocculate.

No influence outside the range of experimental error was exerted on the absorptiometer readings by variation in pH of the reaction mixtures tested between 4·6 and 7·0, or by the use of acetate or citrate-phosphate buffers (0·0001 N) in the liquid as used in the absorptiometer or by such concentrations of maltose and sodium chloride as could be present under the experimental conditions.

Hydrolysis of mixtures of amylose and amylopectin by β-amylase. Table 1 shows absorptiometer readings taken during the reaction calculated to the basis of 5 mg. of residual polysaccharide per 100 ml. The rise in values for the 608 and 607 filters indicates increase in blueness and also that the unhydrolysed residue is becoming richer in amylose and poorer in amylopectin. Decrease in redness, i.e. in the 603 and 602 filter values, due to the loss of amylopectin is obscured by a greater increase due to the relative increase in amylose. The amylose-iodine complex, although characterized by its high blue values, nevertheless yields higher values with the 603 and 602 filters than its equivalent of amylopectin. The most significant figure is the ratio 608/602, an independent indication in that it takes into account the 602 filter readings and at the same time is independent of errors arising from the determination of percentage hydrolysis. The 607 and 603 filter values were throughout this work confirmatory of those of 608 and 602 respectively, but are not quite so distinctive. This ratio rises, but after 33 % hydrolysis it falls again, indicating that the enzyme is no longer liberating maltose from amylopectin faster than from amylose.

Table 1. *Extinction coefficients of unhydrolysed residue during hydrolysis of amylose-amylopectin mixture by β-amylase*

(Extinction coefficients are Spekker absorptiometer readings calculated to a basis of 5 mg. of residue + 10 mg. of iodine in 100 ml. Reaction mixture: in 100 ml., 0·25 g. of amylose, 0·75 g. of amylopectin, 0·8 mg. of soya β-amylase, pH 7, temp., 30°.)

Time (hr.)	Hydrolysis (%)	Ilford filters				Ratio
		608	607	603	602	608 : 602
0	0	0·720	0·770	0·530	0·460	1·57
1	22·7	0·800	0·845	0·537	0·470	1·70
2	33·0	0·850	0·900	0·545	0·448	1·90
4	36·7	0·815	0·870	0·520	0·423	1·89
21	43·8	0·764	0·826	0·533	0·426	1·79
74	46·7	0·704	0·770	0·525	0·413	1·71

Table 2. *Progress of hydrolysis of amylose-amylopectin mixture and of potato starch paste*

(Conditions of reaction as for Table 1.)

(1) Time (hr.)	(2) Residual polysaccharide (%)	(3) Fraction of amylose in residue (x)	(4) Residual amylose (product of columns (2) and (3))*	(5) Residual amylopectin*	(6) Amylose lost by hydrolysis*	(7) Amylopectin lost by hydrolysis*
			Amylose-amylopectin mixture			
0	100	0·256	25·6	74·4	—	—
1	77·3	0·308	23·8	53·5	1·8	20·9
2	67·0	0·344	23·0	44·0	2·6	30·4
4	63·3	0·322	20·4	42·9	5·2	31·5
21	56·2	0·290	16·3	39·9	9·3	34·5
74	53·3	0·250	13·3	40·0	12·3	34·4
			Potato starch paste (1%)			
0	100	0·211	21·1	78·9	—	—
0·25	88·4	0·229	20·3	68·1	0·8	10·8
1·0	64·8	0·275	17·8	47·0	2·3	31·9
1·5	59·3	0·298	17·7	41·6	3·4	37·3
2·0	57·9	0·279	16·1	41·8	5·0	37·1
3·0	56·6	0·264	14·9	41·7	6·2	37·2
6·0	52·8	0·232	12·2	40·6	8·9	38·1
25·0	47·6	0·132	6·3	41·3	14·8	37·6

* As percentage of polysaccharide in original mixture.

The absorptiometer value using the 608 filter for 5 mg. of starch recorded in Table 1 is 0·720, or 0·144 for 1 mg. From the calibration data 1 mg. of amylose would give 0·390 and 1 mg. of amylopectin 0·060. If we take x and $(1-x)$ for the weights of amylose and amylopectin respectively in 1 g. of starch, then $0{\cdot}390x + 0{\cdot}060\,(1-x) = 0{\cdot}144$, whence $x = 0{\cdot}255$. The corresponding figure calculated from the 607 filter values (0·350, 0·086, 0·154) is 0·257, and the mean of the two is 0·256. In this way the figures recorded in Table 2 (upper half), column 3, were calculated.

It will be seen from the last two columns that in the early stages amylopectin was disappearing over ten times as fast as amylose, but later the process was put into reverse. Hydrolysis of potato starch paste (Table 2, lower half) was similar, the small difference being easily accounted for. The check in amylopectin hydrolysis occurred earlier in the artificial mixture because there was less amylopectin in it. When 34·5% of amylopectin (column, 7 upper half) or 37·3% (lower half) had gone, hydrolysis of that constituent almost ceased and the rate of maltose formation as a whole declined sharply.

The behaviour of potato and other starch pastes, including Lintner soluble starches, under a number of conditions was investigated and three examples are given in Table 3, which shows the quantities of amylose and amylopectin disappearing, calculated as above. In the case of the soluble starch the blue colour values for amylose needed for this calculation were taken from readings on amylose made from the same starch.

It was observed that the ratio of rates of maltose formation from amylopectin/amylose is (*a*) about 20 for potato starch at pH 4·6 and 30°, (*b*) reduced by more alkaline pH, (*c*) varyingly reduced when soluble starch is substituted, (*d*) unaffected by concen-

Table 3. *Course of hydrolysis of amylose and amylopectin*

(Results show the amounts of amylose (AML) and amylopectin (AMP) which have disappeared at various times, expressed as percentage of original starch. 1·0% of starch in medium. 608/602 refers to ratio of absorptiometer readings with 608 and 602 Ilford filters, cf. Table 1.)

Nature of starch	Potato			Potato			Soluble (A.R.)		
pH of medium	4·6			7·0			4·6		
Temp. of medium ...	30°			40°			30°		
Enzyme, mg./100 ml. ...	Barley, 20			Soya, 0·8			Soya, 1·6		
Time (hr.)	AML	AMP	608/602	AML	AMP	608/602	AML	AMP	608/602
0	—	—	1·64	—	—	1·68	—	—	1·59
0·25	0·7	11	1·72	—	—	—	1·9	12	1·45
0·5	—	—	—	—	—	—	4·9	21	1·41
1·0	1·5	32	1·84	3·4	26·5	1·88	8·9	33	1·42
1·5	—	—	—	—	—	—	13	36	1·20
2·0	2·3	37	1·80	5·2	33	1·77	16	36	1·01
3·0	3·3	37	1·72	7·0	34	1·67	17	37	0·99
5·0	—	—	—	9·4	36	1·65	—	—	—
6·0	4·9	39	1·73	—	—	—	19	37	—
24	12	39	1·39	18	37	0·9	20	37	0·93
48	—	—	—	21	37	—	—	—	—
144	18	40	0·80	—	—	—	—	—	—

tration and source (barley, soya, yeast) of β-amylase, (e) unaffected by alkaline dispersion of potato starch instead of boiling.

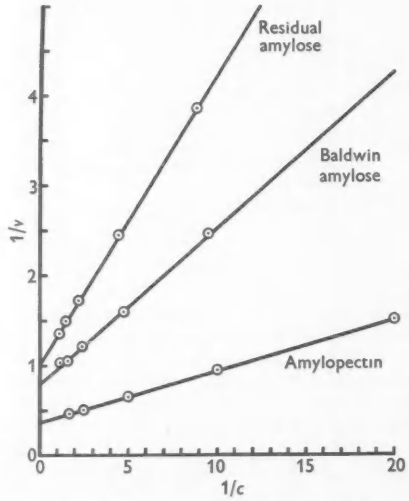

Fig. 2. Initial velocity-substrate concentration relationship plotted according to the procedure of Lineweaver & Burk (1934) for the system soya β-amylase-amylose and amylopectin. Units: v, mg. of maltose formed per min. in 100 ml. of reaction mixture; c, percentage amylose or amylopectin in original reaction mixture. For sources of amylose, see p. 319.

Determination of Michaelis constants of amylose and amylopectin. The procedure described by Hopkins *et al.* (1948) was used with these preparations, but the numerical values obtained may not safely be compared with those reported by these authors. In the present work soya β-amylase acting at pH 5·8 and 30° was employed. In Fig. 2 the graphs of the reciprocals of reaction velocity are plotted against reciprocals of substrate concentration and yield straight lines, thus showing that both kinds of amylose and also amylopectin conform in their hydrolysis by soya β-amylase to the Michaelis-Menten theory. The graph for amylopectin III almost coincided with that of amylopectin II which is shown in Fig. 2. The values calculated from these results were:

	'Baldwin amylose'	Residual amylose	Amylopectin II	Amylopectin III
Soya β-amylase, pH 5·8, 30°	—	—	—	—
Michaelis constant, K_m (%)	0·22	0·32	0·15	0·18
Maximum velocity, V	1·26	1·0	2·67	2·94

The relative rates of decomposition of two substrates A and B, present in equal concentrations and competing for the same enzyme, are given by the formula (Haldane, 1930)

$$\frac{\text{Rate for } A}{\text{Rate for } B} = \frac{V_A}{V_B} \times \frac{K_B}{K_A} = K \text{ (selectivity constant),}$$

where V_A and V_B are the respective maximum velocities, and K_A and K_B the respective Michaelis constants. Taking the mean values for amylopectin and those of the residual amylose (the main constituent), then, in equimixtures of the two,

$$\frac{\text{Rate of hydrolysis of amylopectin}}{\text{Rate of hydrolysis of amylose}} = \frac{2{\cdot}80}{1{\cdot}0} \times \frac{0{\cdot}32}{0{\cdot}165} = 5{\cdot}4.$$

In starch, with nearly 4 parts of amylopectin to 1 part of amylose, the ratio of rates of hydrolysis is much higher.

Relative rates of hydrolysis of different preparations of amylopectin. The preparations I, II and III, described earlier, were submitted to treatment and hydrolysis as summarized in Table 4. The newly prepared sample hydrolysed 1·4 times as fast as the corresponding sample which had been stored for 4 months, but precipitation, etc., of the latter made no difference.

Table 4. *Hydrolysis of potato amylopectin by β-amylase*

(Results as percentage of theoretically equivalent maltose. Reaction mixture: amylopectin, 0·2%; soya β-amylase, 0·4 mg./100 ml.; pH 5·8; temp., 30°.)

Method of preparation ...	From 63° infusion (see p. 319) I	Thymol precipitation. Stored 4 months in sterile solution with thymol II	As II, but stored only 8 days III	As II, but subsequently precipitated with ethanol, dried, stored 1 day IV	As IV, but stored in butanol 1 day V
10 min.	—	6·7	8·7	6·8	6·6
20 min.	15·4	13·8	17·4	13·9	13·4
30 min.	18·4	18·1	23·0	17·8	17·8
24 hr.	51·0	46·4	49·6	46·3	46·4
120 hr.	52·0	50·2	50·1	50·2	48·5

DISCUSSION

The chain length of potato amylose reported in the literature ranges from 200 upwards. The figures are far from certain owing to the considerable effect of any experimental error in the end-group assay on the result of the calculation (Brown, Halsall, Hirst & Jones, 1948). Meyer, Bernfeld, Boissonnas, Gürtler & Noelting (1949), using other methods, report 300 for potato amylose, and Potter & Hassid (1948) 930 and 980 by methods based on viscosity and periodate oxidation respectively.

If it be assumed that β-amylase can attack a non-reducing end group of amylopectin or of amylose with equal facility and hydrolyse either chain to produce maltose at the same rate, then we should expect amylopectin to yield maltose $300/25 = 12$ times (say) as fast as amylose under identical conditions. In starch, which contains approximately 4 parts of amylopectin to 1 part of amylose, about 48 parts of maltose would be derived from amylopectin for 1 from amylose. If the relationship remained unaffected by the diminishing chain length, the amylopectin would be transformed into its limit dextrin (α-amylodextrin), corresponding to about 50% hydrolysis, by the time that only 0·8 part of amylose had disappeared.

However the experiments with potato starches at pH 4·6 and a consideration of the Michaelis constants of amylose and amylopectin led alike to the conclusion that β-amylase splits maltose from the amylopectin component of starch paste about 20 times as fast as from the amylose. This ratio is maintained until about 20–25% of the theoretically equivalent maltose has been split off. The Michaelis constants also indicate that the soya amylase has only about twice the affinity for amylopectin, notwithstanding its possession of some 8–20 times as many terminal points per unit of weight as amylose. This could be due to the close proximity to one another of the end groups in the amylopectin molecule which could not all be attached simultaneously to the large enzyme molecule. Alternatively, the shortness of the branches and complexity of the amylopectin molecule may impede the attachment.

Both in starch paste and in separate solution amylopectin hydrolysis is almost linear with time, whereas that of amylose soon declines in rate (Tables 2 and 3; Hopkins *et al.* 1948). The consistent behaviour of a constituent in paste, alone or in mixtures, supports the view that starch is composite, with which not all workers agree (Le Corvaisier, 1948).

The complete removal of amylose from starch was only achieved at pH 7, under which conditions the rate of hydrolysis of amylose was faster. These conditions, pH 7 and 40°, would be those most suitable for the preparation of α-amylodextrin.

The amylose of Lintner starches was hydrolysed as much as one-third as fast as the amylopectin; this was found also with this particular amylose in separate solution. Presumably a certain amount of hydrolytic fission of the amylose had occurred during the acid treatment in the production of the starch, resulting in a great increase in the molarity and rate of hydrolysis of amylose in the corresponding reaction mixtures.

A similar case is that of potato starch macerated with water at 63° which yields an infusion containing about 5% of the original starch, of which about 4·5% is amylose and 0·5% amylopectin. This amylose, presumably composed of relatively short chains which have made their exit from slightly swollen granules, hydrolyses much faster than normal amylose in solutions of 0·2% concentration,

owing to its higher molarity. The amylopectin which accompanies it into the infusion, amylopectin I, gave much lower blue values with iodine than the other preparations, and indeed resembled in this respect the best preparation otherwise obtained. It can scarcely be representative of the amylopectin of potato starch, and presumably consists of the smaller or less complex molecules.

It is worthy of note that infusion of potato starch for 15 min. at 63°, followed by a further 15 min. at 45–50° before centrifuging, led to the separation of fractions of both amylopectin and amylose, which differ from the main portions of these substances.

SUMMARY

1. β-Amylase splits maltose from the amylopectin constituent of potato starch about 20 times as rapidly as from the amylose.

2. Its reactions with both amylose and amylopectin conform to the Michaelis and Menten formulations, the values of K_m (expressed as percentage) being about twice as great for amylose as for amylopectin.

3. The hydrolysis of an artificial mixture of 4 parts of amylopectin and 1 part of amylose follows the same course as the hydrolysis of starch.

4. The results support the accepted view that starch paste contains these ingredients in unmodified form.

5. From structural considerations it might be expected that amylopectin would be hydrolysed even more rapidly than was found to be the case.

6. The rate of hydrolysis of amylopectin is to some extent influenced by its mode of preparation.

7. The amylose which diffuses from slightly swollen potato starch granules at 63° is hydrolysed much faster than normal potato amylose, while the amylopectin which accompanies it gives an unusually low blue value with iodine.

8. The amylose of Lintner starches is hydrolysed much faster than normal amylose, it having presumably been split in the acid treatment and its molarity in solutions correspondingly raised.

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On the Mechanism of Secretion of Ions by Gastric Mucosa and by Other Tissues

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In this paper the theory of gastric secretion, discussed by Crane, Davies & Longmuir (1948*a*), is extended and certain new evidence is presented.

All the explanations of ionic secretion so far proposed have been more or less incomplete. Franck & Mayer (1947) have examined the general mechanisms required for an 'osmotic diffusion pump'; Rosenberg (1948) has discussed the thermodynamic relationships which must hold for secretory processes; Stiehler & Flexner (1938) related the secretion of chloride into the cerebrospinal fluid to maintained differences of electrical potential and of redox potential across the choroid plexus; Hollander (1943) proposed that the separation of gastric hydrochloric acid is due to differential permeability to ions. None of these authors have related secretion to the activity of particular physico-chemical systems within the cell.

More specific proposals have been made by Crane *et al.* (1948*a*) and Crane & Davies (1948), who have also reviewed a number of other suggestions of the chemical mechanism involved. They distinguished

two types of mechanism. In the first of these, also suggested by Robertson & Wilkins (1948*a*, *b*) to account for secretion by plant cells, an oxidation-reduction system (such as Fe^{++}-Fe^{+++}) produces hydrogen ion (H^+), by oxidation of substrate hydrogen, and OH^- in equivalent amount by reduction of O_2; this mechanism cannot, however, produce more than four H^+ per O_2 used in oxidation and is insufficient to account for the large yields of acid by gastric mucosa. A second type of mechanism was therefore proposed which should transport H^+, derived from water, at the expense of 'metabolic energy'. Recently, Patterson & Stetten (1949) have proposed a mechanism of gastric secretion which is essentially the first mechanism of Crane *et al.* (1948*a*).

The two mechanisms mentioned above are now further examined, in the light of past evidence and of new evidence, presented here, concerning the permeability of gastric mucosa. This evidence was obtained by measuring the potential difference between pairs of electrodes, of three different types, in electrical contact with the solutions bathing the two sides of the mucosa. Specific proposals are made as to the way in which the 'metabolic energy', for the second mechanism, is produced and applied to secretion; the thermodynamic status of each mechanism, and of their interrelationship, is discussed; the electrochemical properties of gastric mucosa are accounted for in terms of the secretory process and of the permeability of the mucosa to ions. The conclusions reached are applied to other types of ionic secretion and to the question of energy-coupling in cellular processes.

EXPERIMENTAL

Methods

The materials, apparatus and methods used in these experiments were similar to those previously described by Davies (1948*a*, *b*) and by Crane *et al.* (1948*a*, *b*).

Frog (*Rana temporaria temporaria* L.) gastric mucosa was mounted in a Perspex holder held between two chambers at 25·0°. The Type II vessels of Crane *et al.* (1948*a*) were used. The potential differences between electrodes were measured with a battery-operated Marconi pH meter and potentiometer, Type TF. 511C: this instrument discriminates better than 0·02 pH or 1 mV. and is accurate to 0·03 pH or 2 mV. Three types of electrode were used: saturated calomel electrodes (Marconi type TM. 3887 A), which were connected to the 'nutrient' and 'secretory' solutions by saturated KCl bridges with internal ground-glass joints (Crane *et al.* 1948*a*); glass electrodes (Marconi type TM. 3888 A), dipped directly into the two solutions; and silver-silver chloride electrodes of the type described by Brown (1934) dipped directly into the two solutions.

Direct measurements were made of the potential differences between pairs of electrodes, except in the case of the glass electrodes, whose combined resistance was too high to allow satisfactory measurements to be made: therefore the differences were taken of the readings obtained between glass in the secretory solution and calomel in the secretory solution, and between glass in the nutrient solution and calomel in the secretory solution. In the course of each experiment, all readings were frequently repeated to ensure that no drifting of the electrodes or deterioration of the mucosa was occurring. All changes of composition were made in the secretory solution, the nutrient solution being, throughout the experiments, frog-bicarbonate Ringer. The concentration of Cl^- in the secretory solution was varied by replacing NaCl with $NaNO_3$, so that the concentration of Na^+ was kept constant. The pH of the secretory solution was varied by the presence or absence of bicarbonate in the secretory solution and by gassing it with 5% CO_2–95% O_2 or with O_2.

In the second experiment, only the total concentration of NaCl in the secretory solution was varied, other factors remaining constant; this was designed to test the effect of the concentration of Na^+ on the potential differences.

Results

The results are not given in full but an abstract of the conditions and readings are given in the tables. Table 1 summarizes the results of the first experiment. Under the headings of 'AgCl-AgCl' and

Table 1. *Potential differences, measured across resting mucosa, with variation of* $[Cl^-]$ *and* pH *of the secretory solution*

(Nutrient solution $[Cl^-]$ = 0·095 M; pH is 7·4 throughout.)

Secretory solution		Potential difference (electrode in nutrient solution = 0 mV.) mV.				
		Calomel-calomel	AgCl-AgCl		Glass-glass	
$[Cl^-]$ *M*	pH		Observed	Calculated	Observed	Calculated
0·095	7·4	−22	−20	−20	—	−20
	7·50	−19, −20	−15	−20	−23, −24	−26
	7·81	—	—	—	−40, −39	−44
	7·89	—	—	—	−41	−49
	8·40	−18, −19	−18	−20	−77, −79	−79
0·012	5·25–5·5	−22	+30	+33	+93	+98
	5·55	−22	+29	+33	+90	+89
	5·60	−22	+34	+33	+84	+86
0·0012	5·50	−22	+91, +87, +90	+92	+88	+92

'glass-glass' are given the observed differences of potential and also those calculated by the equations:

$$\text{P.D.} = 59{\cdot}1 \log \frac{[Cl^-] \text{ (non-secretory side)}}{[Cl^-] \text{ (secretory side)}} - 20 \text{ mV.},$$

and

$$\text{P.D.} = 59{\cdot}1 \, [\text{pH (non-secretory side)} - \text{pH (secretory)}] - 20 \text{ mV.}$$

The agreement throughout between the observed and predicted values is reasonably close.

THEORETICAL

General conditions required for the secretion of ions

'Secretion' of ions here implies the net movement of electrolyte from lower to higher chemical potential.

A single type of ion cannot be secreted alone to any detectable extent, since it is necessary that electrical neutrality should be maintained on both sides of the secreting structure, by the equivalent movement either of ions of opposite sign in the same direction or of ions of the same sign in the opposite direction.

To effect secretion, it is necessary that a constraint of some sort should be applied to at least one of the ions transported and the ion to which such a constraint is applied may be said to undergo 'primary' secretion. The consequent movement of other ions, essentially electrolytic in nature (to maintain electrical neutrality) may be termed 'secondary'. The primary secretion cannot be simply electrolytic, in a wholly aqueous medium; for the charge separation needed to cause electrolytic migration to occur must itself be a result of the previous transport of ions.* Thus neither the maintenance of a difference of redox potential in a metallic system, nor a Donnan distribution across a membrane, can lead to continuous secretion (cf. Hollander, 1943; footnote to p. 417).

Primary secretion must therefore involve the generation of the ion from a precursor in which it is chemically bound, either *de novo*, or following the previous formation of the precursor from the free ion and a carrier substance at some other site. In the former case, the chemical energy required may be resident in the precursor; in the latter it must be made available either at the formation or breakdown of the precursor, or both. These two mechanisms are illustrated in Fig. 1.

It follows that primary secretion can involve only such ions as are capable of being bound chemically,

* Even 'electron transfer', as through a ferrous-ferric system, is equivalent to the transport of ions, since one positive ionic charge necessarily appears for each electron lost.

Table 2. *Potential differences, measured across resting mucosa, with variation of* $[Na^+]$ *of the secretory solution*

(Nutrient solution: $[Cl^-] = 0{\cdot}095$ M; $[Na^+] = 0{\cdot}11$ M; $[K^+] = 0{\cdot}0045$ M.)

Secretory solution		Potential difference mV.			
			AgCl-AgCl		
$[Na^+]$ M	$[Cl^-]$ M	Calomel-calomel	Observed	Expected	Difference
0·12	0·12	−43	−47	−49	—
0·012	0·012	−44, −40, −38	+ 5	+12	− 7
0·0012	0·0012	−50	+32	+71	−39
0·12	0·12	−30	−34	−36	—

Table 2 summarizes the results of an experiment designed to measure the effect of changing the concentration of Na^+ in the secretory solution on the 'asymmetry' potential of the mucosa. It had been noted in previous experiments that the potential difference measured between calomel electrodes tends to become more negative if $[Na^+]$ is reduced as well as $[Cl^-]$ in the secretory solution. The column 'AgCl-AgCl expected' gives the potential difference which should have been obtained between silver chloride electrodes, were the only controlling factor (as in Table 1) the value of $[Cl^-]$ in the secretory solution: the column 'Difference' gives the difference of the observed value from this expected value. In both cases, this difference is negative, by 9 mV. and by $(39 - 7) = 32$ mV. for the two intervals of concentration of Na^+. It is possible, in view of the changing state of the mucosa, revealed by the change of calomel-calomel and silver chloride-silver chloride potential differences on return to 0·12 M-sodium chloride, that the values of these differences are too high, but it seems clear that an increasingly negative 'asymmetry potential' is obtained as the concentration of Na^+ on the secretory side is lowered.

In an earlier experiment, the sodium chloride solution on the secretory side was replaced successively by isotonic sodium nitrate, sodium sulphate, potassium chloride, calcium chloride and phosphate buffer (pH 7·3): the potential difference between calomel-potassium chloride electrodes was in each case the same, within a few mV.; the potential between calomel-potassium chloride electrodes was also independent of changes in the pH of the nutrient solution.

and this is likely to exclude Cl^-, Na^+, K^+ and probably Ca^{++} from primary secretion. Examples of substances and processes which might play a part in primary secretion are: (1) H^+, by reduction to hydrogen atoms and their combination with a carrier; (2) phosphate, by esterification; (3) NH_4^+, by amination or amide formation as ammonia; (4) HCO_3^- by combination of carbon dioxide by carboxylation or formation of a carbamino compound. The last three may also involve, indirectly, the transport of H^+.

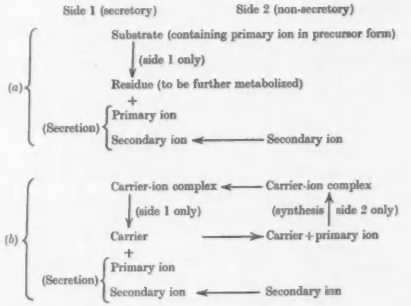

Fig. 1. General schemes showing the mechanisms of secretion (*a*) when the primary ion is derived from a substrate, and (*b*) when the primary ion is initially uncombined.

The ion which undergoes primary secretion need not, in the final state, undergo net secretion, since it may be replaced, after undergoing primary secretion, by exchange with another ion of the same sign: this may be regarded as a 'tertiary process'. In this way it is possible for an electrolyte to be secreted without either of its ions undergoing primary secretion. For example, the primary secretion of H^+, with secondary secretion of Cl^-, followed by tertiary exchange of H^+ and Na^+ (across a structure impermeable to Cl^-), would result in the net secretion of sodium chloride.

Some selective permeability of the structure across which secretion is to take place must always be postulated; it must be relatively impermeable to the primary ion in its free state, so that a rapid backward leakage does not occur, and selectively permeable to the secondary ion.

Gastric secretion

Summary of evidence

This summary is derived from evidence presented in this paper, and by Davies (1948*a*, *b*), Crane *et al.* (1948*a*, *b*) and Crane & Davies (1948).

The main experimental facts which any theory must account for are:

(1) The mucosa can secrete acid continuously for long periods, doing secretory work at a rate of the order of 10,000 cal./g. mol. of HCl.

(2) The secretory process depends upon respiration and is interfered with by any agent which inhibits respiration.

(3) The value of the ratio of g. mol. of HCl secreted to g. mol. of extra O_2 used during secretion ($q_{HCl}/|q_{O_2}|$) may be as high as 11 and such a value may be maintained for a considerable period of time.

(4) For each mol. HCl secreted, there is normally an uptake, on the non-secreting side of the mucosa, of one mol. CO_2, leading to the formation of one mol. HCO_3^-. This process probably requires the activity of carbonic anhydrase (Davies & Roughton, 1948; Davies & Edelman, 1948).

(5) (*a*) The resting mucosa can maintain a potential difference between identical electrodes in identical solutions on the two sides of the mucosa for long periods, even when current is allowed to pass freely between the electrodes in an external circuit. Thus the resting mucosa is capable of the continuous performance of electrical work. (*b*) The potential difference between two calomel electrodes across the resting mucosa is constant and is usually 20–30 mV., the secretory side being negative in the external circuit. The potential difference between two silver-silver chloride or between two glass electrodes, varies systematically with the concentration of Cl^- and H^+, respectively, in the secretory solution, but in both cases there is included an asymmetry potential of the same sign and magnitude as is observed with calomel electrodes. The magnitude of this asymmetry potential varies with the concentration of alkali-metal cation on the secretory side.

(6) Passage of current through the mucosa between zinc-zinc acetate electrodes increases or decreases the secretion of acid by a secreting mucosa according as the applied current augments or diminishes the spontaneous potential measured between calomel electrodes on either side of the mucosa.

(7) Histamine can cause the change between resting and secreting states to occur. When secretion begins, there is an increase of oxygen uptake and a decrease of the asymmetry potential.

Mechanisms of secretion

It is simplest to assume that H^+ is the primary ion and that Cl^- is secreted secondarily. One possible mechanism (mechanism 1) for primary secretion of H^+, by oxidation of the hydrogen of substrate, has already been mentioned; although this is quantitatively inadequate, there is no reason to suppose that it does not occur, but it must be supplemented by another (mechanism 2).

Mechanism 1. The essentials of this mechanism are shown in Fig. 2. A redox system is required which can transfer electrons across a structure which

separates two regions of the cell: this system oxidizes hydrogen of substrates to H^+ and reduces an equivalent amount of oxygen to OH^-, these processes occurring on opposite sides of the structure which is impermeable to H^+, OH^- and to HCO_3^- formed from it. The substance oxidized by this system is likely not to be the ultimate substrate of the cell, but rather a

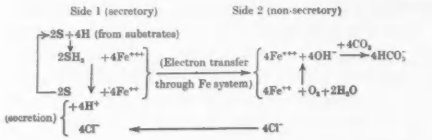

Fig. 2. Scheme of the secretion of HCl by mechanism 1, where the hydrogen is derived from the substrate.

carrier SH_2, such as a flavoprotein; the most likely redox system is cytochrome *c* and cytochrome oxidase. Thus, the reactions are as follows, the subscripts indicating in which region they occur:

Oxidation of SH_2 on side 1:

$$\underset{(1)}{2SH_2} + \underset{(1)}{4Fe^{+++}} \rightarrow \underset{(1)}{2S} + \underset{(1)}{4H^+} + \underset{(1)}{4Fe^{++}}.$$

Oxido-reduction between sides 1 and 2, with transport of Cl^- to maintain electrical neutrality:

$$\underset{(1)}{4Fe^{++}} + \underset{(2)}{4Fe^{+++}} + \underset{(2)}{4Cl^-} \rightarrow \underset{(1)}{4Fe^{+++}} + \underset{(2)}{4Fe^{++}} + \underset{(1)}{4Cl^-}$$

Reduction of O_2 on side 2:

$$\underset{(2)}{4Fe^{++}} + \underset{(2)}{O_2} + \underset{(2)}{2H_2O} \rightarrow \underset{(2)}{4Fe^{+++}} + \underset{(2)}{4OH^-}.$$

Reaction of OH^- with CO_2: the reaction of OH^- with CO_2 takes place in the cytoplasm of the acid-secreting cell, according to the reactions (Davies & Roughton, 1948):

$$\underset{(2)}{4H_2O} + \underset{(2)}{4CO_2} \rightarrow \underset{(2)}{4H_2CO_3} \rightarrow \underset{(2)}{4H^+} + \underset{(2)}{4HCO_3^-}$$

$$\underset{(2)}{4H^+} + \underset{(2)}{4OH^-} \rightarrow \underset{(2)}{4H_2O}.$$

Once a steady state of secretion and of exchange of HCO_3^- with Cl^- of the nutrient fluid (i.e. with blood *in vivo*) is established, these reactions occur *effectively* at the pH of the nutrient fluid.

The overall reaction is:

$$\underset{(1)}{2SH_2} + \underset{(2)}{O_2} + \underset{(2)}{2H_2O} + \underset{(2)}{4Cl^-} + \underset{(2)}{4CO_2} \rightarrow \underset{(1)}{2S} + \underset{(1)}{4H^+} + \underset{(1)}{4Cl^-} + \underset{(2)}{4HCO_3^-}. \quad (1)$$

Since H^+ and OH^- must be produced exclusively on opposite sides of the structure, it follows that the cytochrome system must be oxidizable by oxygen only on side 2, and that SH_2 must be oxidizable by the cytochrome system only on side 1. Equation (1) shows that mechanism 1 cannot produce more than $4H^+$ per O_2, whatever the source of the hydrogen.

Mechanism 2. Since mechanism 1 exploits fully the power of the oxidative system to form H^+ from the hydrogen of substrates, and since no anion other than OH^- (or HCO_3^-) is formed, it follows (Crane *et al.* 1948*a*) that any second mechanism must secrete preformed H^+, that is, H^+ derived from hydrogen atoms in water by ionization. It is again simplest to assume the primary secretion of H^+. The energy of dehydrogenation reactions is not likely to be directly available, but can be made available in the form of unstable phosphate compounds, such as creatine phosphate (see Ogston & Smithies, 1948).

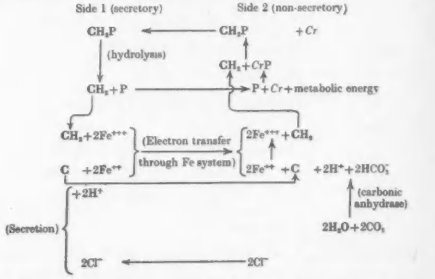

Fig. 3. Scheme of the secretion of HCl by mechanism 2, where the hydrogen is ultimately derived from water.

The essentials of mechanism 2 are shown in Fig. 3. A carrier substance **C** undergoes the reaction, on side 2:

$$\underset{(2)}{\mathbf{C}} + \underset{(2)}{2H^+} + \underset{(2)}{2Fe^{++}} \rightleftarrows \underset{(2)}{CH_2} + \underset{(2)}{2Fe^{+++}}. \quad (2a)$$

If, as is likely, the standard redox potential of the carrier system is considerably lower than that of the ferric system, no considerable amount of CH_2 can be formed by reaction (2*a*). However, phosphorylation of CH_2 by creatine phosphate (*Cr*P), on side 2:

$$\underset{(2)}{CH_2} + \underset{(2)}{CrP} \rightleftarrows \underset{(2)}{CH_2P} + \underset{(2)}{Cr} \quad (3)$$

can cause considerable accumulation of CH_2P, provided that the free energy of breakdown of *Cr*P is sufficiently greater (more negative) than that of CH_2P.

The accumulation of CH_2P on side 2 causes it to diffuse across the separating structure to side 1. Here it loses phosphate with liberation of CH_2, which then undergoes reaction (2*a*) in the reverse direction.

$$\underset{(1)}{CH_2P} \rightarrow \underset{(1)}{CH_2} + \underset{(1)}{P} \quad (4)$$

$$\underset{(1)}{CH_2} + \underset{(1)}{2Fe^{+++}} \rightarrow \underset{(1)}{\mathbf{C}} + \underset{(1)}{2H^+} + \underset{(1)}{2Fe^{++}} \quad (2b)$$

C and phosphate diffuse back to side 2. The ferric system transfers electrons across the structure:

$$\underset{(2)}{Fe^{+++}} + \underset{(1)}{Fe^{++}} \rightarrow \underset{(2)}{Fe^{++}} + \underset{(1)}{Fe^{+++}} \quad (5)$$

and Cl^- diffuses from side 2 to side 1.

The immediate source of the H^+ ions removed from side 2 can be any or all of the acid components

of the cytoplasmic buffer system (e.g. water, protein, H_2CO_3, $H_2PO_4^-$, etc.). Once a steady state of secretion is established, the uptake of carbon dioxide and formation of HCO_3^- balance the removal of H^+, whose ultimate source is therefore water. The formation of HCO_3^-, as in mechanism 1, occurs effectively at the pH of the nutrient fluid (blood).

Thus the total reaction is:

$$\underset{(2)}{2H_2O} + \underset{(2)}{2CO_2} + \underset{(2)}{2Cl^-} + \underset{(2)}{CrP} \rightarrow \underset{(1)}{2H^+} + \underset{(1)}{2Cl^-} + \underset{(2)}{2HCO_3^-} + \underset{(2)}{Cr} + \underset{(2)}{P}. \quad (6)$$

In this scheme it must be postulated that the separating structure is relatively impermeable to CH_2, as compared with CH_2P and **C**; otherwise reaction (2*b*) could occur on side 2 instead of on side 1. An alternative postulate would be that CH_2P does not simply hydrolyse on side 1, but that the enzyme on side 1 which catalyses the reverse of reaction (2*a*) is specific for CH_2P, so that,

$$\underset{(1)}{CH_2P} + \underset{(1)}{2Fe^{+++}} \rightarrow \underset{(1)}{C} + \underset{(1)}{2H^+} + \underset{(1)}{P} + \underset{(1)}{2Fe^{++}}. \quad (2c)$$

In either case, CH_2P must be degraded relatively slowly on side 2.

Thermodynamic relationships

In this section the quantitative data used are those quoted by Ogston & Smithies (1948), unless reference is made to other sources.

General. The free energy required to secrete hydrochloric acid at a concentration isotonic with blood is of the order of 10,000 cal./mol. (Crane & Davies, 1948). The free energy of oxidation of glucose by atmospheric oxygen under physiological conditions is about −58,000 cal./atom of oxygen. Thus the thermodynamic maximum value of $q_{HCl}/|q_{O_2}|$ would be about 12.

Ogston & Smithies (1948) have concluded that the total free energy of oxidation of substrates is divisible into two parts according to the oxido-reduction mechanism which produces it and its position in the scale of redox potentials; the free energy liberated in the transfer of hydrogen to the cytochrome system ($E'_h < 0{\cdot}26$ V.) can lead to the phosphorylation of creatine, while that available from oxidation within the cytochrome system (E'_h between +0·26 and +0·82 V.) is not known to lead to phosphorylation. This division fits in well with the two postulated mechanisms of secretion.

Mechanism 1. The free energy available from an oxido-reduction process is measured by the interval of redox potential over which the process occurs; an interval of 1 V. corresponding with the evolution of 23,100 cal./g. electron. In this case it is supposed that oxidation occurs between atmospheric oxygen at +0·82 V. (the potential of the oxygen electrode at pH 7·0) and cytochrome *c*, at its standard redox potential, $E'_h = +0{\cdot}26$ V. at pH 7·0: this interval of 0·56 V. is equivalent to an evolution of 12,900 cal./g. electron, that is, per g.ion of H^+ and this is amply sufficient for the secretory process.

Mechanism 2. The immediate source of energy is the hydrolysis of creatine phosphate: its standard free energy at 37° is −16,700 cal./mol. and this is unlikely to be very different at 25°; but at a concentration of phosphate of 14 mM., and assuming a ratio of $CrP/Cr = 1$, the actual free energy becomes −19,000 cal./mol.*

Assuming that, in reaction (6), the reaction

$$\underset{(2)}{H_2O} + \underset{(2)}{CO_2} \rightleftarrows \underset{(2)}{HCO_3^-} + \underset{(2)}{H^+}$$

is at equilibrium and neglecting activity coefficients since the secretion is isotonic with blood, the free energy change in reaction (6) is

$$\Delta F_{(6)} = RT \ln \frac{[H^+_{(1)}]\,[Cl^-_{(1)}]}{[H^+_{(2)}]\,[Cl^-_{(2)}]} - \frac{1}{2} \times 19{,}000 \text{ cal./mol. HCl}$$

$= 0$ for secretion of HCl at the maximum possible concentration.

Taking $[H^+_{(2)}]$ for the nutrient fluid $= 10^{-7{\cdot}3}$ M and $[Cl^-_{(2)}] = 10^{-1}$ M, this gives at 37°

$$[H^+_{(1)}] = [Cl^-_{(1)}] = 10^{-0{\cdot}79} \text{ or } pH = 0{\cdot}79,$$

which is equivalent to 0·162 M-HCl. This value depends on the values assumed for the pH of the nutrient fluid and for the concentration of phosphate; the value used for the latter is the resting concentration of frog muscle.

Thus the hydrolysis of 1 mol. of *Cr*P is capable of providing enough free energy to secrete 2 mol. of HCl at the order of concentration at which it is produced by oxyntic cells (Babkin, 1944).

The total free energy of oxidation of glucose, less the amount expended by mechanism 1, is 32,000 cal./atom of oxygen. The maximum P/O ratio for phosphorylation of creatine would thus be 32,000/19,000 = 1·7 and the maximum value of $q_{HCl}/|q_{O_2}|$ would be $4 \times 1{\cdot}7 = 6{\cdot}8$.

Some light may be thrown on the components of mechanism 2 by considering its kinetic requirements in relation to thermodynamic quantities. Thus, for the reactions on side 2 to proceed rapidly, the steady-state ratios $[CH_2P]/[C]$ and $[Fe^{+++}]/[Fe^{++}]$ should not differ greatly from unity. If the system on side 2 is further assumed to be near equilibrium, under the conditions stated, then the sum of the free energies of reactions (2*a*) and (3) is zero: this sum is given by the sum of the standard free energies of the phosphorylation processes (free phosphate is not involved in these reactions) and of the oxido-reduction

* Ogston & Smithies (1948), in considering the probable amount of phosphorylation accompanying the oxidation of pyruvate, used the value −16,700 for the free energy of hydrolysis of *Cr*P. Had they taken the concentration of phosphate into consideration, their predicted values of P/O ratio would have been lower still.

processes. Assuming that the standard free energy of hydrolysis of CH_2P is -3000 cal. (i.e. that this is a 'low energy' phosphate compound) and that $\Delta E'_0$ is the difference of the standard redox potentials of the ferric and the $C\text{-}CH_2$ systems, then

$$2F\Delta E'_0 - 16{,}700 + 3000 = 0$$

(F is the Faraday equivalent of electric charge) so that $\Delta E'_0 = 0{\cdot}30$ V. This value would suggest that either cytochrome b ($E'_0 = -0{\cdot}04$ V.: Ball, 1938) and malate-oxaloacetate ($E'_0 = -0{\cdot}17$ V.) or cytochrome c ($E'_0 = +0{\cdot}26$ V.) and fumarate-succinate ($E'_0 = 0$) might suitably act as the ferrous-ferric carrier combination.

Combination of mechanisms 1 *and* 2

Mechanisms 1 and 2 might conceivably act together in three different ways.

(1) In series, such that one supplies H^+ to be further concentrated by the other. The value of $q_{HCl}/|q_{O_2}|$ of this arrangement would be limited to 4, but a concentration of H^+ of the order of 10^{12}-fold could be produced. There is no reason to think that this arrangement occurs in nature. Two secreting structures separating three regions of the cell would be required.

(2) In series, but back-to-back, so that one mechanism supplies H^+ to the secretory side of the other; this is identical with the 'in parallel' arrangement (3), if escape of H^+ from the cell cannot occur. This is an interesting possibility, because, each mechanism being reversible, mechanism 1 could drive mechanism 2 backwards, leading to phosphorylation of creatine and this might explain how oxidation in the cytochrome system at high E'_h could bring about phosphorylation (Ogston & Smithies, 1948). Friedkin & Lehninger (1948) have shown that phosphorylation can accompany the oxidation of dihydrocoenzyme I by cytochrome c, in a liver-particulate system: it is doubtful, however, whether such a degree of organization as would be required by our mechanism could have survived in such a preparation.

(3) In parallel, each mechanism contributing additively to the secretion of H^+. It has been shown that each mechanism is thermodynamically adequate. The sum of their maximum yields ($4 + 6{\cdot}8$) is of the same order as the observed maximum of $q_{HCl}/|q_{O_2}|$. There are, however, certain further limitations on the distribution and reaction of components between the regions of the cell, if the two mechanisms are to work successfully side by side.

(i) The oxidation of hydrogen (i.e. of SH_2) must occur only on side 1 and the reduction of oxygen only on side 2.

(ii) The phosphorylation of creatine, resulting from the transfer of hydrogen from substrate to flavoprotein (**S**), can occur anywhere in the cell, provided that *Cr*P can transfer phosphate to the carrier **C** (mechanism 2) only on side 2.

(iii) The ferrous-ferric system of mechanism 2 must not be accessible to oxidation by O_2 and so must not be coupled with the ferrous-ferric system of mechanism 1: otherwise its redox potential would tend to rise too high to allow sufficient formation of CH_2 on side 2. This consideration would perhaps exclude the cytochrome c-fumarate-succinate system from acting in mechanism 2.

Resting and secreting states

From the facts that the oxygen utilization of resting mucosa is fairly high and that the resting mucosa is capable of developing continuous electrical work in an external circuit (up to 10 % of its total metabolic energy), we assume that the processes which lead to secretion are still active in the resting state, but do not actually lead to secretion. Potential measurements (see below) show that the resting mucosa, as a whole, is effectively impermeable both to H^+ and to Cl^- and the impermeability of the outer membrane of the cell would be a sufficient cause for non-secretion. If the secretory mechanism were still active in the resting state, the result would be to produce an accumulation of HCl within the region of the cell called 'side 1' (Figs. 2, 3). It is reasonable to suppose that the structure across which secretion takes place is not completely impermeable to H^+, so that there would be a continuous backward leakage of H^+ from side 1 to side 2 and the metabolic energy of the cell would be required to maintain a steady-state excess of HCl on side 1.

The onset of secretion must involve the cell outer membrane becoming permeable to H^+ and to Cl^- and the acquisition of this permeability would alone be sufficient to cause secretion to occur. At the same time, the loss of HCl from side 1 of the interior of the cell removes a constraint on the oxidative mechanisms, so that an increase of oxygen uptake would ensue automatically. It is possible, also, that the permeability of the secretory structure to H^+ and to Na^+ (see below) might be reduced at the same time, which would increase the efficiency of secretion; Cope, Cohn & Brenizer (1943) have shown that radioactive Na^+ is absorbed more rapidly by the resting than by the secretory stomach. It seems possible, therefore, that the action of histamine is to affect the permeability of cellular structures and so to allow H^+ to pass out of the cell (cf. Crane *et al.* 1948*a*).

It is of interest, in this connexion, that thiocyanate, in concentrations of 10^{-3} to 10^{-2} M, inhibits both the uptake of iodide by the thyroid (Franklin, Chaikoff & Lerner, 1944) and the secretion of acid by the gastric mucosa (Davenport, 1940; Crane *et al.* 1948*a*); it is possible that both actions are due to the prevention of halide ions from entering cells.

Potential measurements

General

When two electrodes are present in any system and a potential difference between them can be observed, this potential difference represents the electrical work which is

done when a unit of electric charge is allowed to pass between the electrodes. This work must be derived from changes of free energy taking place within the system. When the whole system is reversible, the amount of electrical work can be equated to this change of free energy, but where irreversible changes take place as, for example, through free diffusion at a junction between two ionic solutions, this exact relationship does not necessarily hold good. Also, changes which do not affect the electrodes can occur without contributing to the electromotive force of the cell.

In attempting to interpret potential measurements, it is important to use this approach whenever it is possible to do so, and not to treat the electromotive force of a cell as the sum of a number of 'single potentials' (e.g. potentials of electrode against solution, diffusion or membrane potentials). It has been repeatedly pointed out that such single potentials can neither be measured nor defined and, though an argument based on their use may often lead to the right conclusion, it cannot be relied upon to do so.

Unfortunately, it is the common practice to measure potentials with electrode systems which introduce unnecessary liquid junctions: the ionic transport across such junctions can usually not be calculated, so that it becomes necessary to use the 'single potential' approach.

Valuable information can be obtained about systems which contain membranes selectively permeable to different ions, by the use of simple reversible electrodes. The general conditions which govern the potential difference between a pair of such electrodes are as follows.*

(1) If two phases are separated by a membrane impermeable to all ions and one electrode is in each phase, no definable or reproducible potential is observed.

(2) If the membrane is permeable to some ion or ions and the two phases are at equilibrium with respect to those ions, then (*a*) zero difference of potential is found with electrodes reversible to any ion to which the membrane is permeable; (*b*) a finite and reproducible potential difference is observed with electrodes reversible to an ion to which the membrane is impermeable; as a special case, the potential difference is zero if this ion is distributed as it would be at equilibrium, were the membrane permeable to it. In this case, the potential difference measures the free energy of transfer of electrolyte from one phase to the other, which results from the reaction of the reversible ion at the electrodes.

(3) If two phases are not at equilibrium with respect to ions which can move from one phase to the other, and if passage of current between a pair of electrodes, one in each phase, facilitates the transfer of ions with respect to which the disequilibrium exists, then a reproducible potential is observed. The magnitude of this potential depends upon the mobilities of all ions which are transported when current passes between the electrodes, and can be calculated only in simple cases. (This case (3) is equivalent to 2 (*b*), if the transport number of the ion to which the electrodes are reversible is zero.)

The considerations outlined above do not indicate the sign of the potential difference; to determine this it is necessary to discover in which direction current must pass between the pair of electrodes used so as to diminish the free energy of the system.

* The conditions under which a potential difference is observed between a pair of electrodes in two phases in membrane equilibrium were given incorrectly by Ogston (1947, p. 234) and are here corrected.

Resting gastric mucosa

When resting frog gastric mucosa has identical solutions on its two sides, there is found to be a potential difference between a pair of silver chloride or glass or calomel electrodes of about 30 mV., the secretory side being negative in an external circuit. If the concentration of Cl^- on the secretory side is changed, the potential difference between a pair of silver chloride electrodes is independent of pH and is closely given by

$$\frac{2{\cdot}303\,RT}{F}\log\frac{[Cl^-]\text{ nutrient}}{[Cl^-]\text{ secretory}} - 0{\cdot}03\text{ V.}$$

This shows that the resting mucosa is effectively impermeable to Cl^-: the result of passage of 1 g. ion equivalent of electricity between the electrodes is to transfer 1 g.ion of Cl^- from one side to the other and there is an equivalent transport of Na^+ through the membrane, the concentration of Na^+ being the same on both sides. Similarly, the potential difference between a pair of glass electrodes is independent of the concentration of Cl^- and is given by

$$\frac{2{\cdot}303\,RT}{F}\log\frac{[H^+]\text{ secretory}}{[H^+]\text{ nutrient}} - 0{\cdot}03\text{ V.}$$

It follows that the resting mucosa is also effectively impermeable to H^+.

Patterson & Stetten (1949) measured independently the potential difference across acid-secreting rat gastric mucosa, with glass electrodes and stated: 'It was repeatedly observed that the pH difference, as computed from the total potential difference, and as calculated from individual determinations of pH on both sides of the membrane, agreed within 0·2 pH unit.' This shows that the potential difference between calomel electrodes which was not determined (Stetten, 1949) could not have been more than 12 mV. Such low values of the potential difference between calomel electrodes are usually found with partially anoxic or damaged isolated mammalian gastric mucosa (Davies, 1948*b*).

The variations of potential differences between these pairs of electrodes with composition of the secretory solution represent changes of the free energy of ionic transport between the outer solutions. The constant 'asymmetry potential' of about −30 mV. must represent a process occurring within the mucosa independently of changes in the composition of the secretory solution.

The effective impermeability of the whole mucosa to H^+ and Cl^- is likely to be due to its external membrane. This membrane is permeable to Na^+ and so will behave as a pair of reversible sodium electrodes with respect to the interior of the cell: formation or removal of H^+ or Cl^- by the external electrodes can result only in a corresponding movement of Na^+ into and out of the cell. In the case where the external

solutions are identical, the whole electromotive force of the system (-30 mV.) must therefore arise from changes in an electrolytic cell of this type (Fig. 4).

Now, if this represented simply a Donnan distribution of Na^+ and Cl^-, as a result of the presence of a fixed excess of H^+ on side 1, then no difference of potential could arise between the pair of sodium electrodes. But if H^+ can be transferred backwards (whether through the secretory mechanism or by 'leakage' through the separating structure), then the passage of current between the electrodes will affect this transfer; the cell is not in membrane equilibrium but is a 'concentration cell with liquid junction', whose composition is continuously maintained by the secretory mechanism. Equilibrium can be approached by transfer of H^+ from side 1 to

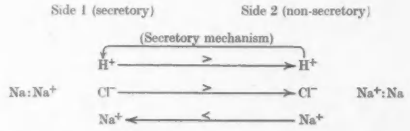

Fig. 4. Diagram of the resting oxyntic cell, regarded as a 'concentration cell, with liquid junction', using reversible sodium electrodes. The sign > indicates differences of concentration between the two sides, and the arrows the directions of movement of ions.

side 2: evidently the addition of Na^+ to side 1 and its withdrawal from side 2 facilitates this transfer of H^+. Thus the condition is satisfied that passage of current between the external electrodes, such that that on the secretory is negative, facilitates the approach to equilibrium. Since this is not a reversible system, and since the values of the mobilities of the ions concerned are not known, no quantitative prediction of the magnitude of this e.m.f. can be made: but it is within the limits commonly observed in 'diffusion potentials' (cf. the values observed by Crane *et al.* 1948*a*, across a cellophan membrane).

The system becomes more complicated if K^+ or Ca^{++} is substituted for Na^+ on the secretory side. The unchanged potential difference observed between calomel electrodes presumably means that these ions are closely similar to Na^+ with respect to their rate of migration through the cellular structure; the mucosa has, in fact, been found to be easily permeable by a variety of cations, including those of dyes (Visscher, 1942; Höber, 1945).

Reduction of the concentration of Na^+ in the secretory solution has been shown to increase the magnitude of the asymmetry potential. This is explained if the concentration of Na^+ within the oxyntic cells is thereby lowered, for this will decrease the transport number of Na^+ and increase that of H^+, resulting in a more negative 'diffusion potential' arising within the cells. Such a reduction of the concentration of Na^+ within the cells would necessarily imply that the cells are somewhat permeable to some anions; but this is not inconsistent with the conclusion, reached above, that the mucosa is effectively impermeable to H^+ and to Cl^-, since this means only that it is far less permeable to these ions than to Na^+ or K^+.

Secreting gastric mucosa

When the mucosa passes into the secreting state, the outer walls of the cells must become permeable to H^+ and to Cl^-. Since the increase of H^+ in the outer fluid is normally accompanied by an equivalent increase of Cl^-, the process of tertiary exchange of H^+ against Na^+ must be relatively slow. But it is not necessary that there should be a decrease of permeability to Na^+; the relatively high electrical resistance of the resting mucosa shows that this is never very great. The rate of back-diffusion of H^+ through the secreting structure likewise need not be great. The removal of hydrochloric acid from the interior of the cell will tend to reduce the 'diffusion potential' across the secreting structure, so that, as is observed, the potential differences between external calomel electrodes should fall.

The effect of an external source of current on the rate of secretion of active mucosa is probably simply electrolytic in nature: with the secretory electrode negative, the system will behave as a Hittorff cell, provided that the transport number of H^+ through the mucosa is greater than 0·5, and will concentrate hydrochloric acid on the secretory side. The failure to obtain electrolytic formation of acid using resting mucosa and other membranes (Crane *et al.* 1948*b*) was probably due to their low permeability to H^+.

Other types of ionic secretion

Although the evidence about other types of ionic secretion is less complete, it seems likely that all will show features in common. (For reviews see Ussing, 1949*b*; Davies, 1948*b*.) Thus where a neutral salt is secreted it is possible, in most cases, to assume that an ion such as H^+, NH_4^+, HCO_3^- or $H_2PO_4^-$ may undergo primary secretion and may then effect a tertiary exchange with another cation or anion, as the case may be (cf. Kinsey, 1949; Davson, Duke-Elder, Maurice, Ross & Woodin, 1949; Lundegårdh, 1945; Robertson & Wilkins, 1948*a*, *b*; Hoagland, 1948). This hypothesis makes no demand upon unknown sorts of chemical process, though it does imply a mosaic arrangement or an alternating function of the structures concerned. Similarly, it is possible to modify the interpretation put by Ussing (1949*a*) upon his experiments with frog skin by assuming that the transport of Na^+ is secondary to that of H^+, rather than the reverse.

The secretion of acid and the absorption of bicarbonate by the tubules of the kidney (Pitts, 1945; Pitts & Lotspeich, 1946*a*, *b*; Pitts, Ayer & Schiess, 1948) could occur by the gastric mechanism, a greater flexibility with regard to anions being due either to a variable secondary secretion or to a variable tertiary exchange. The secretion of bicarbonate by the pancreas is likely to depend on the gastric mechanism working in reverse (Davies, 1949).

The transport of ions between the interior and exterior of cells, across a single-cell membrane, appears to be the simplest case. Conway & O'Malley (1946) showed that yeast may secrete H^+ into the external medium in exchange for K^+ and the 'acidic region' which they postulate, and the membrane separating this from the rest of the cell, might well correspond with the 'side 1' and the 'secreting structure' of our model. In other cases, as where Na^+ appears to be actively extruded, there is insufficient evidence to decide whether its primary secretion must be assumed.

SUMMARY

1. Evidence is presented of the differences of potential observed between pairs of calomel, silver chloride and glass electrodes when one of a pair is in the 'nutrient' solution, the other in the 'secretory' solution, of isolated, non-secreting gastric mucosa of the frog. The values of these potential differences and their variation with composition of the secretory solution have been interpreted in terms of the permeability of the mucosa to ions.

2. An extended theory of the chemical and physicochemical mechanism of gastric secretion has been developed, in the light of present knowledge of the chemistry and thermodynamics of cellular processes. This is shown to be consistent with the evidence available concerning gastric secretion.

3. In the light of this theory, some other cases of ionic secretion are discussed.

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Displacement Chromatography on Synthetic Ion-exchange Resins

5. SEPARATION OF THE BASIC AMINO-ACIDS

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In Part 1 of this series of papers (Partridge & Westall, 1949) it was shown that, whilst the base-exchange resin Zeo Karb 215 may be used with success for the separation of the neutral and acidic amino-acids, certain difficulties arise when the separation of basic substances is attempted. These difficulties were traced to the phenolic hydroxyl groups of the resin. Resins of the sulphonated phenol-formaldehyde type are multifunctional when in equilibrium with solutions of high pH. Titration curves (Partridge & Westall, 1949, Fig. 4) show that over the range pH 1–7 the sulphonic acid groups only are dissociated, but when the pH rises from 7 to 12 the phenolic hydroxyl groups also dissociate and thus over this range the resin retains cations on both types of group.

Measurement of the boundary widths of fronts due to the retention of sodium ions from a range of buffered solutions of different pH showed that sharp boundaries were obtained over the range pH 1–7, but that under more alkaline conditions the boundaries became progressively wider (Partridge & Westall, 1949, fig. 7). This suggested that the rate of exchange between sodium and hydrogen ions was rapid under acid conditions when the sulphonic acid groups only were involved, but was much slower when phenolic hydroxyl groups also played a part.

Other experiments showed that the effect was also important when one base displaced another from the resin, and successful separations were obtained only with components of lower basic strength than ammonia; in the experiments on the fractionation of a protein hydrolysate, described in Part 3 of this series (Partridge, 1949*a*), ammonia was used as the displacement developer, in spite of the fact that this reagent was insufficiently basic to displace arginine. In practice it was found that the affinity of ammonia for the resin was not much greater than that of lysine and thus this amino-acid also was not readily displaced and could not be recovered quantitatively. The difficulty with which arginine and lysine are recovered from a column of Zeo Karb 215 either by elution with hydrochloric acid or by displacement with sodium hydroxide has been stressed by Hems, Page & Waller (1948), who were unable to obtain satisfactory recoveries of these two amino-acids in experiments with protein hydrolysates.

In order to overcome this difficulty experiments have now been carried out with samples of another type of resin which contained no phenolic hydroxyl groups. This was a resin of the sulphonated crosslinked polystyrene type. As will appear below, attempts to use a commercial sample of this resin resulted in failure, and for this reason variously modified samples of sulphonated crosslinked polystyrene resin were prepared in the laboratory.

It soon became apparent that the rate of exchange of large organic bases varied considerably with the degree of crosslinking of the resin, and sharp boundaries were only obtained on resin samples in which the structure was relatively open. However, resins of open structure suffered from the corresponding disadvantage of excessive shrinkage with increases in the ionic strength of the solution phase, and it was necessary to take special steps to minimize disturbance of the boundaries due to this cause.

EXPERIMENTAL

Preparation of sulphonated crosslinked polystyrene

The preparation of sulphonated crosslinked polystyrene (sulphonated PSX) involves two stages, first the copolymerization of styrene and divinylbenzene in bead form, and secondly sulphonation of the resulting copolymer (D'Alelio, 1942). Styrene and divinylbenzene, together with benzoyl peroxide (1% w/v) as polymerization catalyst, were shaken vigorously at room temperature with a 2% (w/v) aqueous dispersion of 'Promulsin' (a cellulosic derivative obtained from the Watford Chemical Co.). The hydrocarbon phase dispersed in the weak gel, forming spherical droplets which were sufficiently stable to allow polymerization to be effected at 80° for 18 hr. without further agitation. The product, after removal of 'Promulsin' by acid hydrolysis (2 N-H_2SO_4 at 100° for 4 hr.) followed by washing, consisted of regular spheres.

The copolymer was sulphonated by heating with excess conc. H_2SO_4 in presence of Ag_2SO_4 as catalyst at 100° for

8 hr. The product was poured into cold water and washed thoroughly by decantation. It was then treated alternately with 2 N-NaCl and 2 N-HCl (three cycles), washed with water, air-dried and sieved.

Determination of swelling and shrinkage

Sulphonated PSX is a hygroscopic swelling gel. The moisture regains of a typical sample at 32% and 75% R.H. and at saturation were 0·2, 0·6 and 1·5 g. of water per g. of dry hydrogen resin. The observed regain increases with decreasing degree of crosslinkage. The volume shrinkage observed with increase in concentration of ambient solution is equivalent to desorption and is also dependent on the degree of crosslinkage.

Regain at saturation was measured by weighing a saturated sample after removal of excess liquid water by centrifuging. A sample of the resin in the hydrogen form (0·5 g.) was placed in a weighed filter tube, and the whole immersed in water at 20° for 1 hr. (absorption of water is complete in a few min.). The filter tube was closed with a rubber cap and centrifuged at 2000 rev./min. for 24 hr. The temperature was maintained at 20° throughout by running the centrifuge in an air thermostat. After weighing, the saturated sample was dried at 105° *in vacuo* and reweighed.

Approximate values for shrinkage resulting from increase in ionic strength were obtained by a column method. Hydrogen resin (10 g.) were backwashed with distilled water in a graduated column. The volume of the column of resin was noted after allowing to settle for 1 hr. with tapping. Corresponding settled volumes were then obtained after backwashing with HCl solutions of different strengths.

Determination of titration curves

Sulphonated PSX was regenerated with 2 N-HCl and washed until the effluent was free from chloride and had a pH in the range 4–5. Samples of air-dry resin (0·5 g.) were weighed into a series of 100 ml. flasks and at the same time samples were taken for determination of moisture content. To each of the flasks were added different amounts of NaOH keeping the solid/liquid ratio approximately constant (75 ml. of solution/0·5 g. of solid). A parallel series of experiments was made in presence of NaCl as added neutral electrolyte. After shaking for 24 hr., the pH of the equilibrated solution was determined with a glass electrode, using a Cambridge Alki electrode for pH's >9. Finally, 25 ml. samples of solution were titrated for acid liberated or exess base.

The amounts of Na^+ ion retained (in mmol. per g. of dry hydrogen resin) are plotted against pH in Fig. 1.

Properties of resins used

In all, four samples of sulphonated PSX were examined, three prepared in the laboratory and one a commercial product.

The degree of crosslinkage of the samples prepared in the laboratory was systematically varied by varying the proportion of divinylbenzene copolymerized with styrene (Table 1).

The capacity of each of the three products was independent of particle size and corresponded with the value calculated for a monosulphonic acid of crosslinked polystyrene. Table 1 demonstrates the marked reduction in swelling and shrinkage which results from increased crosslinking.

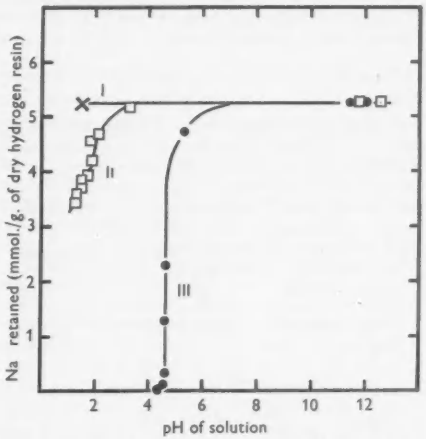

Fig. 1. The effect of pH on the Na^+ retained by sulphonated crosslinked polystyrene: I, in the presence of 5 M-NaCl; II, in the presence of 0·1 M-NaCl; III, in the absence of added salt.

The commercial sample (capacity = 5·0 mmol. per g. of dry H^+ resin) had a saturation regain of 0·75 g. of water per g. of dry resin and volume shrinkages slightly smaller than Chemical Research Laboratory sample no. 2169. It was apparently more highly crosslinked than any of the materials prepared in the laboratory.

Exploratory separation experiments suggested that the highly swelling samples offered the best prospect of success, hence sample no. 2088 was chosen for a more detailed investigation of physical properties and ion-exchange characteristics. The results obtained are summarized below.

Effect of pH *on the retention of sodium ions*

It is seen from Fig. 1 that the amount of Na^+ taken up by sulphonated PSX is independent of pH, at least for all pH values >1·5, provided there is a large excess of Na^+ in solution. The exchange reaction may be represented by

$$HR + Na^+_{solution} \rightleftharpoons NaR + H^+_{solution},$$

where HR denotes sulphonated PSX.

Table 1. *Properties of laboratory-prepared sulphonated crosslinked polystyrene*

Chemical Research Laboratory reference no.	Nominal content of divinylbenzene (%)	Capacity (mmol./g. of dry H^+ resin)	Saturation regain (g. of water/g. of dry H^+ resin)	Volume shrinkage of H^+ resin in 1 N-HCl (%)	2 N-HCl (%)	5 N-HCl (%)
2088	4	5·30	1·80	10	15	25
3168	5	5·30	1·47	5	12	20
2169	10	5·25	0·82	0	4	10

If the resin behaves as a strong acid and is fully ionized at all pH's, the amount of Na^+ taken up at equilibrium depends solely on the affinity constant and the ratio $[Na^+]/[H^+]$ in solution. Provided this latter ratio is large and no other ionizable group is present, the uptake of Na^+ must be independent of pH and equal to the total capacity. The lower values obtained at low pH's in absence of added salt or with 0·1 N-NaCl are due to the ratio $[Na^+]/[H^+]$ being < 100.

The behaviour of sulphonated PSX is entirely consistent with that expected of a unifunctional, strongly acidic, exchanger (Hale & Reichenberg, 1949).

The retention of amino-acids and bases by a sample of sulphonated polystyrene resin

The procedure adopted for the determination of ϵ (the amount of base retained by the hydrogen form of the resin expressed as mmol./g.) has already been described in detail for the absorption of bases and amino-acids by Zeo Karb 215 (Partridge & Westall, 1949) and, since the same procedure was followed in the present work, no further description need be given. The air-dried Na resin was graded by sieving in the dry state and the 80–120 mesh/in. fraction was used for packing the column.

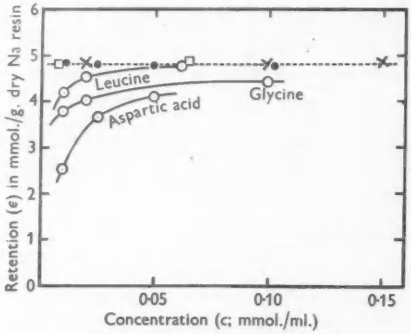

Fig. 2. The retention of a number of bases and amino-acids on sulphonated PSX resin no. 2088 (80–120 mesh/in.). Dotted line, arginine - -□- -, ammonia - -×- -, NaOH - -●- -.

Fig. 2 shows values of ϵ obtained for a range of amino-acids of varying isoelectric point from aspartic acid (pI, 2·77) to arginine (pI, 10·76). The curves are plotted in the conventional form as distribution isotherms and show the amount (mmol.) of solute taken up by 1 g. of resin from pure aqueous solutions of various concentrations. Since the resin was normally stored in the Na form the results are given in terms of the weight of the resin in its dry Na condition and, for convenience, this convention has been adopted throughout the remainder of the paper. The retention volumes in frontal analysis of the solutions of NH_3, NaOH and arginine were measured by titration of successive fractions of the effluent with standard acid. Aspartic acid was determined by titration with NaOH and leucine by formaldehyde titration. Electrical conductivity readings were used for the estimation of glycine.

It will be observed that the isotherms of NH_3 and NaOH are almost horizontal over the concentration range 0·01–0·15 M, showing that the retention over this range is stoicheiometric. The isotherm of arginine falls on the same straight line in the range 0·01–0·06 M. Thus, when the amino-acid is taken up from isoelectric solutions of this concentration it behaves stoicheiometrically as a univalent base. The neutral amino-acid, leucine, is taken up almost to the same extent as arginine from concentrated solution, but at dilutions below 0·05 M the retention falls off rapidly.

The shape of the titration curves of the resin (Fig. 1) suggests that aspartic acid (pI, 2·77) and glutamic acid (pI, 3·22) should be rather slightly retained from isoelectric solution, and the isotherm of aspartic acid (Fig. 2) shows this to be the case. Glycine (pI, 5·97) occupies an intermediate position between aspartic acid and leucine.

Boundary widths of fronts formed on sulphonated PSX resin by various bases

The method used for the determination of boundary widths was that given in Part 1 of this series (Partridge & Westall, 1949). Table 2 shows the results of a number of determinations with various bases and amino-acids. Resin sample no. 2088 was used for these experiments, and the determinations were carried out with a column of 9·0 mm. diameter and 92 mm. height, containing 1·7 g. (dry wt.) of the resin.

Table 2. *The width of various boundaries on sulphonated polystyrene resin columns*

(4% Divinylbenzene, 80–120 mesh/in.)

Boundary	Rate, S (cm./hr.)	Boundary width, λ (cm.)
NaOH	12·5	1·5
	3·9	0·5
Ammonia	5·3	1·0
	11·3	1·2
Arginine	1·5	1·8
	8·9	1·5
Leucine	3·2	1·5
	9·1	1·7
Glycine	9·8	0·7
	20	1·1
Aspartic acid	5·3	1·7
	10·6	1·9

The determinations refer to the widths of the boundaries of the fronts due to solutions of the bases on a column packed with the resin in its acid form, and thus the results give a qualitative indication of the readiness of displacement of H^+ by other cations. The widths measured were of the same order for all the bases and amino-acids and were generally rather lower than those observed with samples of Zeo Karb 215 of similar particle size. Since mechanical packing defects in the column frequently give rise to oblique fronts and these prevent the observation of boundaries narrower than 0·5–1·0 cm., the dependence of the boundary width (λ_{90}, as defined by Partridge & Westall, 1949) on the rate of progression of the front down the column (S) is not brought out clearly by the data in Table 1; but as in the case of Zeo Karb 215, it is clear that no real advantage is to be obtained by employing progression rates slower than 10–15 cm./hr. Variation of the concentration of the inflowing solutions over the range 0·01–0·1 M had no effect on the boundary widths observed.

Recovery of arginine and lysine from columns of sulphonated crosslinked polystyrene resin

Arginine was not readily eluted by dilute HCl and quantitative elution required large volumes of acid. When a column containing 1·7 g. of resin sample no. 2088 was saturated with arginine it was found that while 84% of the amino-acid was eluted by passage of 50 ml. of N-HCl, no less than 250 ml. was required to elute 98%. Higher concentrations of HCl were more effective and elution of 99% of the arginine held on the column was obtained by passage of 45 ml. of 3N-HCl. However, the displacement of arginine by NaOH proceeded smoothly and quantitatively, sharp boundaries being formed between the arginine and NaOH bands. In one experiment an aqueous solution (75 ml.) of arginine monohydrochloride (1·58 g.) was allowed to pass through a column containing 1·7 g. (dry wt.) of the resin (sample no. 2088). The column was then rinsed with a small quantity of water and the arginine displaced by passage of 0·15N-NaOH, the rate of flow being adjusted to establish the migration of the Na^+ boundary at about 10 cm./hr. The effluent was collected in 10 ml. fractions and the displacement followed by paper chromatography. The arginine in the effluent solution was then estimated by a modification of the Sakaguchi method (Devine, 1941) and showed a recovery of 99%.

No difficulty was encountered in the recovery of lysine from columns of sulphonated PSX by displacement with solutions of NaOH, provided that development was carried out without delay. However, in chromatograms carried out with the hydrolysis products of egg albumin the presence of a number of peptides of lysine has been observed, and it is not clear whether this was due to a synthetic reaction catalysed by the resin or to the presence in the hydrolysate of a small residue of resistant material. The presence of such peptides in similar circumstances was observed by Block (1947), and in this case the evidence appeared to point to resynthesis.

The effect, as we have observed it, may be illustrated by reference to a particular experiment in which the more basic amino-acids of a protein hydrolysate were allowed to remain on a column of sulphonated PSX at 0° for 10 days. The chromatogram obtained by displacing the mixture with 0·2N-NaOH showed the presence of a small amount of peptide material. This was concentrated at the leading edge of the lysine band, and a filter-paper chromatogram (solvent: butanol-acetic acid mixture) showed it to consist of four distinct components (R_F: (*a*) 0·37, strong spot; (*b*) 0·33, weak spot; (*c*) 0·25, strong spot; (*d*) 0·04, weak spot). Further paper chromatograms were cut so as to isolate the spots due to the individual compounds; the cut pieces were then extracted with water and the eluates hydrolysed with 5·5N-HCl at 100° for 30 hr. Further chromatography of the hydrolysis products showed that all the fractions contained lysine and that the two stronger spots *a* and *c* contained respectively leucine and valine in addition to lysine.

The presence of such peptides has not been observed in chromatograms of mixtures of amino-acids of known purity, and it cannot be excluded that they may have been present, as such, in the hydrolysate of the protein; it may be significant that the peptides appeared to be very resistant to hydrolysis and that digestion with 5·5N-HCl at 100° for 18 hr. was insufficient to complete the reaction.

Packing disturbances due to the shrinkage of sulphonated PSX resin

Although the fronts formed by the passage of solutions of the amino-acids through columns packed with the resin in its acid form had sharp boundaries (cf. Table 2) it was found that when mixtures of the amino-acids were placed on the columns and subsequently displaced by NaOH, only the first amino-acid boundary was sharp and the bands formed by the succeeding components overlapped one another considerably. This behaviour could only be understood in terms of disturbing effects caused by the volume changes taking place in the resin. As the first front traverses the column the resin shrinks owing to the increase in the ionic strength of the solution phase. This does not disturb the first front, but destroys the uniformity of packing in the part of the column already traversed. Subsequent fronts are then distorted by the presence of channels and gaps between the resin and the walls of the filtration tube.

It is clear that such disturbances would be reduced by the choice of an operation cycle in which changes in ionic strength were minimized. In the separation of the more basic amino-acids it is permissible to saturate the column, before use, with a solution of a base weak enough to be displaced by the amino-acids, and a considerable diminution of changes in swelling can be obtained by preliminary saturation of the column with glycine hydrochloride. Accordingly, the following operation cycle was adopted for the separation experiments described below: (1) the column was regenerated with 2N-HCl, (2) it was then washed with 0·1N-HCl, (3) a solution of glycine hydrochloride (0·1M), sufficient to saturate the column, was then applied, (4) the mixture of basic amino-acids was applied at 0·15–0·2M-concentration, (5) displacement was carried out with 0·15–0·2M-NaOH.

A further method by which an improvement in the separations was obtained was by dividing the column into two portions; a long upper part and a short lower part. The disturbance in a short column by changes in the volume of the resin particles is less serious than that in a long column, for in the latter case part of the weight of the column packing is supported by the sides of the filtration tube, and such a column does not resettle so readily under gravity. A short column, placed in position below the main long column, acts as a corrector and increases the sharpness of the boundaries as they pass through it.

Both these expedients appeared to improve the performance of narrow laboratory columns in separating mixtures of the more basic amino-acids and, in the next section, examples are given of separations carried out by these means.

Separation of mixtures of the more basic amino-acids

The object of these experiments was to make a direct comparison of the performance of the various resin samples under as nearly as possible identical conditions. Sample 2169 and the commercial sample did not swell excessively, but nevertheless the same precautions to reduce disturbances due to swelling were taken with these samples as with resins of lower degree of crosslinking. Therefore the columns were made in two sections, and were saturated with glycine hydrochloride as described above.

The construction of the columns was generally similar to that described by Partridge & Westall (1949). Since the

volume occupied by a given weight of resin when packed wet into a column varied to a marked degree with the cross-linking and was different for each of the four resin samples, it was not possible to fix both the dry weight of resin and the height occupied in filtration tubes of the same bore. For the purpose of these experiments the height of the packed column was fixed, and the amount of resin taken was chosen to fill the columns to a fixed height when in equilibrium with 0·1 N-HCl. The main column was in each case 27 cm. high, packed in a glass tube of 11 mm. diameter; and the height of the lower column was 2·5 cm. (diameter 13·5 mm.). Both columns were provided with glass floats to prevent disturbance to the top surface of the resin by the impact of the drops of solution flowing into the tubes. The weight of resin (dry wt., Na form) packed into the two filtration tubes in each case was as follows: sample no. 2088, 8·5 g.; sample no. 2168, 10·6 g.; sample no. 2169, 16·2 g.; commercial sample, 20·6 g.

The columns were regenerated and saturated with 0·1 M-glycine hydrochloride solution as described in the preceding section. The test solution contained L-leucine, 0·784 g.; L-histidine monohydrochloride, 1·148 g.; L-lysine dihydrochloride, 1·315 g.; L-arginine monohydrochloride, 1·263 g. dissolved in water (100 ml.). This was allowed to flow through the column at a rate of 40 ml./hr. Displacement development was carried out with 0·15 N-NaOH, the solution being applied at such a rate as to allow the complete development of the column in 6 hr. (rate of progression of the Na^+ boundary, 5 cm./hr.).

The effluent solution was collected in fractions of equal volume (10 ml.) and a drop from each fraction taken for the preparation of filter-paper chromatograms. The results of the fractionations are shown in Fig. 3.

Fig. 3 (*a*) shows the analyses of the effluent obtained with the most highly swelling resin (no. 2088). Calculation of the boundary widths between the various amino-acid bands show these to be approx. 1 cm. for the two boundaries of the lysine band and approx. 2 cm. for the boundaries of the leucine band. Thus the separation was as sharp as could be expected with a column of over 1 cm. in diameter and it is clear that, with boundaries as sharp as this, excellent recoveries could be obtained by increasing the length of the column and increasing the amount of the amino-acid mixture applied to it.

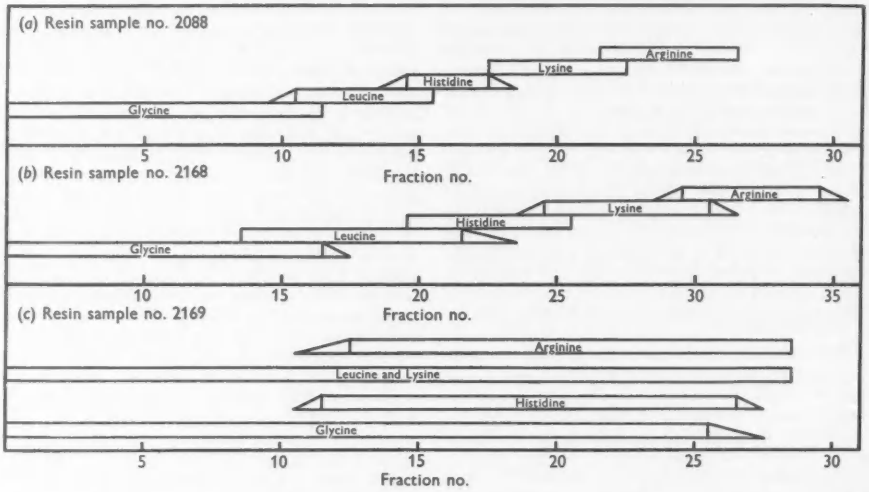

Fig. 3. Chromatogram prepared by displacing with NaOH mixtures of leucine, histidine, lysine, and arginine from columns packed with various samples of sulphonated crosslinked polystyrene resin (40–70 mesh/in.). The columns were treated with 0·1 M-glycine hydrochloride before application of the amino-acid mixture.

Fig. 3 (*b*) shows the chromatogram obtained with resin no. 2168. In this case the boundaries were not as sharp (boundary widths, 3·6–1·8 cm.), but a useful separation was obtained. Boundary widths of the order shown by this resin can well be tolerated with large columns (of 1 in. or more in diameter), and since the sample suffered less swelling than sample no. 2088 it is probable that its use would be preferable in work on a preparative scale.

The chromatogram obtained with sample no. 2169 is shown in Fig. 3 (*c*). Here, no useful separation was obtained, and similar results were given by experiments carried out with the commercial sample, which was even more crosslinked than no. 2169. Since the separations obtained in these and other experiments were so poor, no further work was carried out with either of these resins.

Use of sodium chloride solution as displacement developer

In Part 4 of this series (Partridge, 1949*b*) the effect of using NaCl as the displacement developer instead of a free base was described. Using Zeo Karb 215, a rather remarkable change in the order of displacement was observed, and use was made of the effect to secure the recovery of glucosamine and histidine in a pure condition. The following was the order of displacement of a series of bases from a Zeo

Karb column by dilute NH_3 solution: leucine, histidine, glucosamine, lysine, NH_3. With 0·05–0·10 M-NaCl as displacement developer the order was: glucosamine, Na^+, leucine, histidine, lysine. It was considered of interest to determine if the same reversal of order occurred with the sulphonated PSX resin, particularly as this resin is more generally suitable for use with the stronger bases.

The column was packed with 8·5 g. (dry wt.) of resin sample no. 2088 and was identical with that used in the experiment illustrated in Fig. 3 (*a*). The test solution (100 ml.) contained D-glucosamine hydrochloride, 0·634 g.; L-leucine, 0·392 g.; L-histidine monohydrochloride, 0·567 g.; L-lysine dihydrochloride, 0·637 g. After regeneration, the column was washed with 0·05 N-HCl. Preliminary saturation of the column with glycine hydrochloride was unnecessary since the ionic strength of the solution was maintained by the presence of HCl derived from the base hydrochlorides and from the NaCl solution used as displacement developer. After passage of the test solution, 0·05 M-NaCl (400 ml.) was applied at a rate of 200 ml./hr.; the solution was then replaced by a stronger solution of NaCl (0·10 M, 400 ml.) and the rate slowed to about 150 ml./hr. In order to displace histidine and lysine, the NaCl solution was then replaced by 0·15 N-NaOH which was applied at 120 ml./hr.

The resulting chromatogram is illustrated in Fig. 4 (*a*) which shows that the four components were well separated. The leading boundary of the histidine band was contaminated by a little leucine indicating that the volume of NaCl solution was not quite sufficient to complete the elution of leucine. The position of the Na^+ front is indicated by the curve in Fig. 4 (*b*) which shows that NaCl breaks through at fraction 25, immediately behind the glucosamine rear boundary. The curve shows the concentration of HCl in the effluent and was obtained by titrating successive fractions with standard NaOH solution to the end point of bromthymol blue.

DISCUSSION

The use of sulphonated crosslinked polystyrene instead of the sulphonated phenol-formaldehyde resin used in previous work has proved of particular value for the separation of the three basic amino-acids: histidine, lysine and arginine. This was to be expected since it was considered that the slow rate of exchange of strong bases by the latter type of resin was due to the intervention of phenolic hydroxyl radicals. However, not all samples of sulphonated crosslinked polystyrene resin were effective and a further point of interest has emerged from the study of resin samples prepared in the laboratory in which the degree of crosslinking varied. In exchange reactions involving organic bases of relatively high molecular volume the structure of the polymer network is an important consideration, and such reactions proved to occur more readily with the more highly swelling and less strongly crosslinked samples than with those of closer structure. From this it appears that the relationship between the molecular volume of the solute and the size of the apertures in the resin framework, through which the exchanging cations must pass in both directions, may be critical. A similar conclusion has been reached from studies of the rates of exchange of organic bases of different

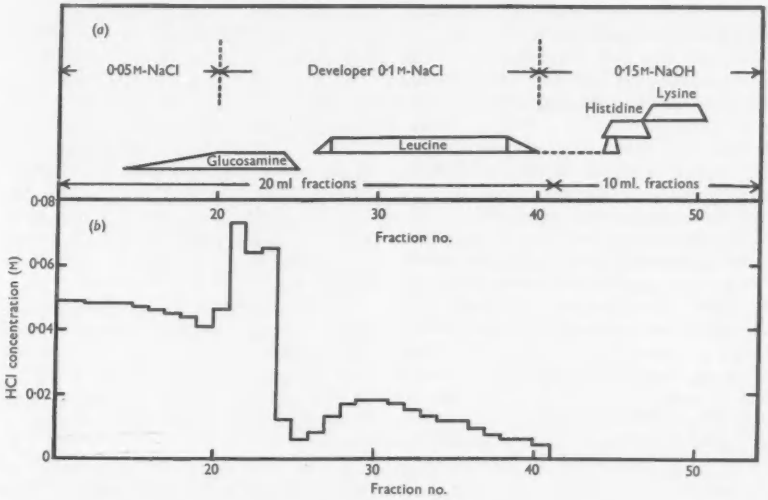

Fig. 4. (*a*) Chromatogram prepared by displacing a mixture of glucosamine, leucine, histidine and lysine from a column of sulphonated PSX resin, first with an increasing concentration of NaCl, followed by 0·15 M-NaOH. (*b*) Concentration of HCl in the effluent. Each fraction was titrated with standard NaOH to the end point of bromthymol blue.

molecular size on a single sample of resin (Kressman & Kitchener, 1949); the effect has not been described previously as a direct consequence of variations in the molecular structure of the resin.

Determination of the widths of boundaries of fronts due to various solutes in columns packed with the resins gives a qualitative picture of the overall rate of the exchange reaction involved, but accurate quantitative results cannot be obtained by this method where the exchange rates are fast and the boundaries correspondingly narrow. The main reason for this is that boundaries are invariably more or less oblique due to mechanical unevenness in the packing of the columns and thus boundary widths of less than 0·5–1·0 cm. are rarely observed in practice. Where the rate of an exchange reaction is slow, as for instance in the case of resins of large particle size, there exists the possibility of correlating the exchange rate and the boundary width more accurately.

Although the more highly swelling resin samples possess the advantage of rapid reaction, they suffer from the corresponding disadvantage of excessive shrinkage with changes in ionic strength. It was observed that disturbance due to this cause was more serious in long narrow columns than in shorter columns of wider bore, and consequently the choice of the most suitable type of resin must depend upon the scale of the experiments envisaged and upon the detailed design of the column. From experience gained in this work it appeared that resins made with 4–5 % of divinylbenzene were suitable for fractionation experiments on a small laboratory scale. However, on the basis of the data so far available, no decision can be made concerning the most suitable resin for preparative work on a larger scale. In order to place the design of larger-scale columns on a rational basis, detailed information on the swelling properties and rates of exchange for a range of resins is required. This information may be obtained more systematically and with greater certainty from the results of static experiments rather than from direct observation of columns. The detailed design of column assemblies of high efficiency must await the collection of such data.

SUMMARY

1. The use of sulphonated phenol-formaldehyde cation-exchange resins in the preparation of displacement chromatograms leads to difficulties in the separation of basic amino-acids. These difficulties are due to the phenolic hydroxyl groups of the resin and an attempt has been made to avoid them by use of a sulphonated crosslinked polystyrene resin containing no phenolic hydroxyl groups.

2. Three samples of polystyrene resin prepared in the laboratory and one commercial preparation were investigated. Variations in the degree of crosslinking produced by variations in the amount of divinylbenzene used in the preparation of the resin gave rise to important differences in the behaviour of the sulphonated product towards organic bases.

3. Two resin samples with a low degree of crosslinking gave sharp boundaries in displacement chromatograms using basic amino-acids, but suffered from the defect of excessive shrinkage with changes in the ionic strength of the solution phase. The most highly crosslinked laboratory sample and the commercial sample did not shrink excessively, but the boundaries observed in displacement chromatograms carried out under comparable conditions were very diffuse.

4. In order to minimize the disturbances of the column associated with excessive shrinkage, two expedients were adopted: (*a*) the operation cycle was adjusted to avoid large changes in the ionic strength of the solution phase; (*b*) the continuity of the column packing was broken near the base of the column.

5. Employing these expedients, mixtures of leucine, histidine, lysine and arginine were separated successfully.

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Water-miscible Solvents in the Separation of Amino-acids by Paper Chromatography

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The separation of amino-acid mixtures into discrete spots on paper chromatograms has hitherto almost invariably been carried out using solvents only partially miscible with water. Our work has entailed the frequent use of *s*-collidine as a developing solvent. This solvent is objectionable chiefly on account of its offensive smell, because of possible toxic effects (Ludwig, 1935; Holtzmann, 1936) and also because it frequently gives 'ghost' spots and double spots which are confusing when dealing with unknown products of protein hydrolysis. In an effort to replace collidine we have examined many other solvents both partially miscible and totally miscible with water. Edman (1946) found that a mixture of equal volumes of pyridine and amyl alcohol had practically the same resolving capacity as collidine and could with advantage replace this solvent. Consden, Gordon & Martin (1944) stated originally that solvents completely miscible with water can be used provided the water content of the solvent is not too high, but that even then the amino-acid bands are too broad to be of value. We have confirmed that this is so, but only when volatile solvents are used to develop the chromatogram by downward irrigation from horizontal troughs in the conventional apparatus (Consden *et al.* 1944; Dent, 1948). In this type of apparatus, with large air space, it is inevitably some time after the fitting of the canopy or the lid before the atmosphere becomes saturated with solvent and water vapour. During this time preferential evaporation of a volatile solvent occurs from the surface of the paper as the water-saturated solvent travels along it. This leaves the paper waterlogged. Goodall & Levi (1947) overcame this difficulty with wet ether in the separation of penicillins by devising an airtight container with a much reduced air space and equilibrating the contents of the apparatus at 0°. Karnovsky & Johnson (1949), for the same purpose, used an airtight container in which equilibration was completed before addition of solvent. This was done through an inlet tube without having to open the main container. Non-volatile water-miscible solvents can, however, be used in the conventional manner, and in a preliminary communication (Bentley & Whitehead, 1949) we have described the successful use of two such solvents. This work has now been extended to include several other solvents which are comparatively volatile and which are satisfactory if run by ascending capillary irrigation in sealed glass tanks (Williams & Kirby, 1948).

Arden, Burstall, Davies, Lewis & Linstead (1948) have used water-miscible solvents for the separation of inorganic ions, and recently Hanes & Isherwood (1949) have used them for the separation of phosphoric esters. On starch columns, Moore & Stein (1949) have separated amino-acids using water-miscible solvents.

The water-miscible solvents employed successfully in the present work include methanol, ethanol, *n*-propanol, acetone, pyridine, tetrahydrofuran, furfuryl alcohol and tetrahydrofurfuryl alcohol. Dioxan failed to give satisfactory spots; veratryl alcohol is insufficiently volatile to be dried easily; allyl alcohol is satisfactory but extremely unpleasant for routine use.

MATERIALS AND METHODS

Solvents. Acetone and pyridine are of A.R. quality. Methanol, ethanol and *n*-propanol are the commercial absolute alcohols. Furfuryl alcohol is freshly distilled *in vacuo* before use, since the clarity of the final chromatographic picture is seriously impaired if the solvent has acquired a yellow coloration; the inclusion of urea (0·5%, w/v) as a stabilizer (Wilson, 1947) sharpens the outlines of the spots and reduces their size. Commercial tetrahydrofurfuryl alcohol and tetrahydrofuran are used without further purification. Phenol used with these solvents in two-way chromatograms is prepared by saturating Liquified Phenol (B.P.) with water.

Apparatus. With furfuryl alcohol and tetrahydrofurfuryl alcohol conventional apparatus is employed. The solvent flows downward from a horizontal trough over a sheet of filter paper ($18\frac{1}{2} \times 22$ in.) suspended vertically and enclosed in a canopy ($30 \times 30 \times 5$ in.) (cf. Consden *et al.* 1944; Dent, 1948). With volatile solvents the method of Williams & Kirby (1948) is used. Amino-acid spots are placed 6 cm. from the shorter edge of a sheet of filter paper ($18\frac{1}{2} \times 20$ in.) and at least 10 cm. from the longer edges where solvent flow is uneven. The longer edges of the sheet are joined with wire staples to form a hollow cylinder. This is placed vertically in a glass tank ($12 \times 8 \times 21$ in.) the floor of which is covered to a depth of approximately 0·5 in. with solvent (about 600 ml.) which can be re-used for several successive runs, but which must be frequently replaced because of changes in composition due to unequal evaporation of water and solvent. The open end of the tank is carefully sealed by means of a

greased glass plate to keep the internal atmosphere saturated with solvent and water vapour. The tanks are kept in a constant-temperature room at 18·3±0·5°.

Paper. Whatman no. 1 paper is used in the glass tanks, when the solvent front reaches a convenient height (30–35 cm.) in about 15 hr. (overnight); furfuryl alcohol and tetrahydrofurfuryl alcohol move at approximately the same rate by downward flow. On Whatman no. 4 paper the solvents move more quickly, reaching a similar point after about 8 hr.

Visualization of spots. The positions of the amino-acid spots are revealed by spraying the dried papers with a solution of ninhydrin (0·1%, w/v) in dry *n*-butanol in the usual manner. In the case of furfuryl alcohol, if the paper is dried at too high a temperature, particularly in the presence of furoic acid, brown stains appear in the area of the spots which then fail to develop the characteristic colours; further, with this solvent rapid fading of the colours occurs and the spots should therefore be marked immediately after development.

RESULTS

Under the conditions described above most of the amino-acids give small, sharply defined spots. With the basic amino-acids, applied as hydrochlorides, the spots are sometimes elongated and lysine and ornithine often give long streaks; where this occurs no R_F value (Consden *et al.* 1944) is included for the amino-acid in Table 1 and Figs. 1 and 2. Edman (1946) and Dent (1948) have already noted this effect in the case of basic amino-acids. Cystine often gives an unsatisfactory spot and tyrosine streaks with furfuryl alcohol-pyridine. The addition of urea (0·5%, w/v) to acetone and furfuryl alcohol improves the definition of the spots, but has little effect on R_F values.

The R_F values for the amino-acids with the various solvents, together with the amounts of water (v/v) and other additions named are given in Table 1. As pointed out by Dent (1948), the values will vary (up to 15%) with the conditions of a particular experiment and according to the type of apparatus used. The relative positions of the spots, however, remain the same, and for each experiment known amino-acids should be used as markers under conditions identical with those used for unknown substances. R_F values obtained with Whatman no. 1 and Whatman no. 4 papers are approximately the same. It will be seen that almost identical results are given by acetone and pyridine; for routine use the former is obviously to be preferred. Additions of pyridine and of furoic acid to furfuryl alcohol give useful variations in R_F values. The addition to acetone of hydrochloric, acetic, trichloroacetic or benzenesulphonic acids either causes excessive streaking or else the amino-acid spots all progress with the solvent boundary.

The effect of increasing water content on the R_F values in furfuryl alcohol is illustrated in Fig. 1. The actual magnitudes of the R_F values, but not the sequence, vary with the amount of water present, increasing with increasing water content whilst the range of values decreases. We have used furfuryl alcohol containing up to 80% water (v/v). At this high level there is little if any separation of the amino-acids and the bands are very broad, but at 60% water content there is some differentiation of R_F values and the spots are reasonably compact. With all solvents streaking occurs to a greater or lesser extent below 20% (v/v) water content and the optimum water content for small, sharply defined spots lies within the range 20–40% (v/v).

Fig. 2, in which the amino-acids are arranged in ascending order of R_F value in water-saturated *n*-butanol, gives a comparison between this solvent and the three lower water-miscible alcohols whose aqueous solutions we have studied. Whilst the general pattern is broadly similar in each case, the amino-acids are seen to move more slowly with increasing molecular weight of the alcohols and the variations in R_F value for different amino-acids become less.

Each of the water-miscible solvents we have used behaves normally in two-way paper chromatograms. Acetone containing water (40%, v/v) has proved particularly useful in our work, replacing collidine as a second solvent for chromatograms run first with phenol. In work connected with the isolation of a toxic factor from NCl_3-treated zein (Bentley, Macdermott, Pace, Whitehead & Moran, 1949) a fraction was obtained giving a single spot on paper chromatograms with *tert*-amyl alcohol, *n*-butanol, *n*-butanol-acetic acid, benzyl alcohol, furfuryl alcohol or phenol. With aqueous acetone three distinct spots with well-spaced R_F values were obtained. Collidine gave a similar pattern, but the occurrence of double spots and haloes with this solvent, as well as the very low R_F values, made interpretation ambiguous. Further purification yielded crystalline material giving a single spot in aqueous acetone, thus affording some evidence for the homogeneity of the material.

DISCUSSION

The fact that typical separations can be effected on paper chromatograms with water-miscible solvents as well as with solvents only partially miscible with water raises the question of the mechanism of paper chromatography as applied to amino-acids. Consden *et al.* (1944) originally postulated a partition mechanism for the paper chromatogram, whereby the solute occupied a position on the chromatogram determined by its partition coefficient between the flowing solvent and the water phase supported in the cellulose fibres. These authors suggested that, in the case of water-miscible solvents containing small amounts of water, a 'salting-out' effect occurred, allowing the system to function as a partition chromatogram. Arden *et al.* (1948) suggested that this explanation was too simple and that other factors were operative, such as selective extraction of the mixture by the solvent at the site of the test spot and adsorption of the material by the paper. Our results agree particularly well with a broader concept of the mechanism of paper chromatography recently developed by Hanes & Isherwood (1949). These authors stress the fact that the water held in filter paper cannot be considered as free liquid water, but is bound in some way, possibly by hydrogen bonding, to the hydrophilic hydroxyl groups of the cellulose. They suggest that the solute molecules, by virtue of their hydrophilic groups, will compete with water and solvent molecules, which usually contain hydrophilic groups also, in the mobile phase for incorporation in this cellulose-water complex. The distribution of any given solute (and hence the R_F value) will depend upon such factors as the number,

Table 1. *R_F values of amino-acids with water-miscible solvents*

(Colours given by ninhydrin, blue (*B*); brown (*Br*); grey (*G*); purple (*P*); yellow (*Y*). The colours given in all the solvents except furfuryl alcohol are the same as with pyridine.)

Solvent	Tetrahydrofuran	Tetrahydrofurfuryl alcohol	Pyridine	Acetone	Acetone	Furfuryl alcohol	Furfuryl alcohol	Furfuryl alcohol	Furfuryl alcohol
Water and other additions (v/v)	40% water	20% water	35% water	40% water	40% water + 0·5% w/v urea	25% water	25% water + 0·5% (w/v) urea	25% water + 8% pyridine	25% water + 5% furoic acid
Paper, Whatman no. ...	1	4	1	1	1	4	4	4	4
Direction of running ...	Ascending	Descending	Ascending	Ascending	Ascending	Descending	Descending	Descending	Descending
Aspartic acid	0·38	0·15	0·43 *B*	0·45	0·38	0·18	0·20 *B*	0·07	0·23
Glutamic acid	0·45	0·19	0·48 *P*	0·51	0·46	0·18	0·23 *P*	0·09	0·33
Cystine	—	0·20	—	—	—	0·16	0·19 *G*	0·06	0·11
Serine	0·48	0·34	0·51 *P*	0·47	0·44	0·20	0·29 *P*	0·14	0·26
Glycine	0·44	0·28	0·41 *RP*	0·45	0·42	0·21	0·28 *Br*	0·15	0·29
Ornithine*	0·30	0·31	0·29 *P*	0·16	0·19	0·24	0·28 *P*	0·10	0·24
Arginine*	0·34	0·27	0·31 *P*	0·15	0·20	0·34	0·32 *P*	0·17	0·32
Histidine*	0·59	0·31	0·43 *GP*	0·38	0·33	0·25	0·39 *Br*	0·22	0·31
Lysine*	—	—	—	—	—	0·25	0·20 *P*	0·13	0·28
Alanine	0·67	0·43	0·54 *P*	0·55	0·52	0·34	0·39 *PB*	0·26	0·47
Threonine	0·66	0·46	0·56 *P*	0·57	0·50	0·35	0·34 *P*	0·22	0·37
Hydroxyproline	0·63	0·39	0·56 *Y*	0·53	0·54	0·35	0·41 *Y*	0·32	0·43
Tyrosine*	0·72	0·59	0·65 *GP*	0·63	0·69	0·46	0·47 *G*	—	0·52
Valine	0·73	0·64	0·65 *P*	0·62	0·65	0·50	0·52 *BP*	0·43	0·64
Methionine	0·66	0·67	0·66 *P*	0·63	0·65	0·55	0·58 *P*	0·42	0·59
Proline	0·73	0·49	0·56 *Y*	0·58	0·62	0·56	0·56 *Y*	0·42	0·61
Tryptophan*	0·78	0·70	0·63 *Br*	0·58	0·64	0·56	0·59 *GP*	0·43	0·61
Leucine	0·78	0·72	0·68 *P*	0·69	0·71	0·59	0·63 *P*	0·43	0·69
Isoleucine	0·78	0·71	0·66 *P*	0·67	0·71	0·59	0·63 *P*	0·43	0·70
Phenylalanine	0·79	0·73	0·66 *P*	0·64	0·67	0·63	0·64 *GP*	0·52	0·72

* These amino-acids were put on the papers as hydrochlorides.

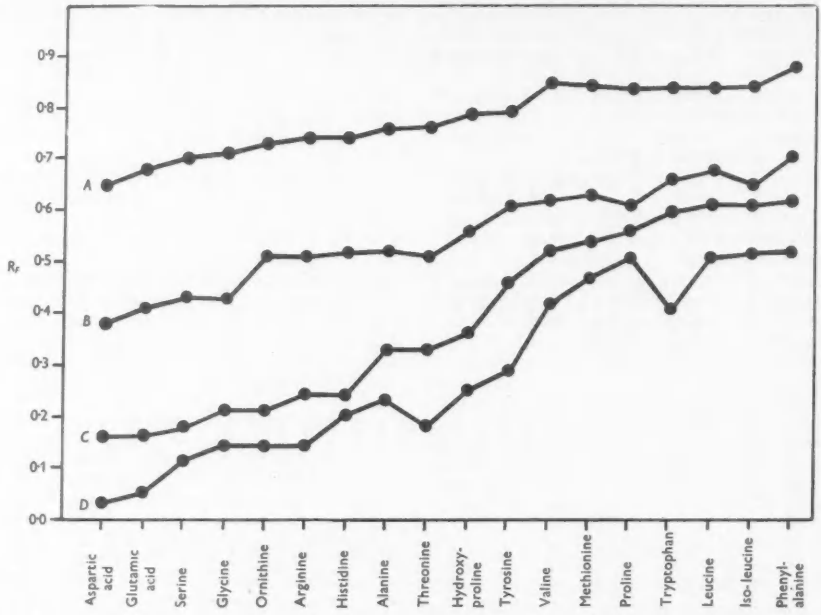

Fig. 1. Effect of water content of furfuryl alcohol on the R_F values of amino-acids on Whatman no. 4 paper at room temperature. (Water content (v/v): *A*, 60%; *B*, 40%; *C*, 30%; *D*, 10%.)

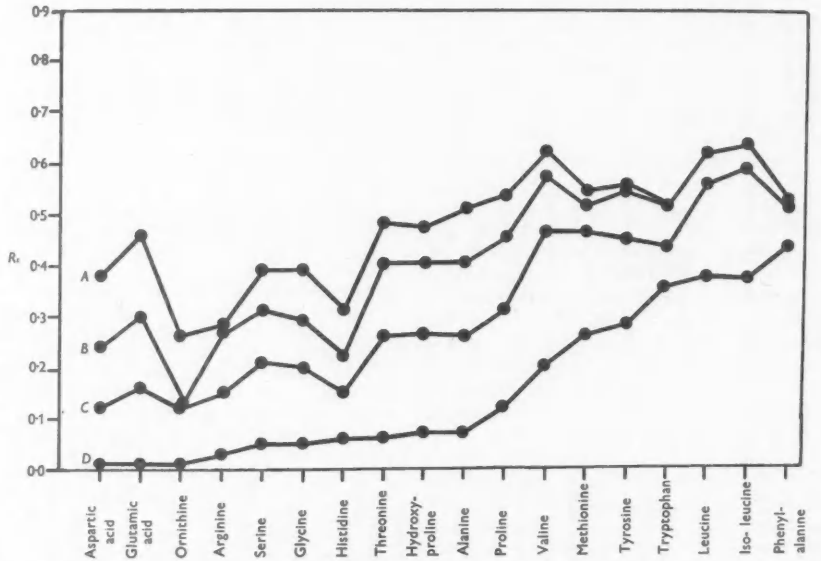

Fig. 2. Comparison of the R_F values of amino-acids in aqueous lower alcohols on Whatman no. 4 paper at room temperature. (*A*, 70% (v/v) methanol; *B*, 70% (v/v) ethanol; *C*, 70% (v/v) *n*-propanol; *D*, *n*-butanol saturated with water.)

position and character of the hydrophilic groups which it contains and also on the water content and the number, position and character of the hydrophilic groups of the solvent.

On this hypothesis it would be expected that, for a given solvent, an increase in water content would result in the solute molecules moving more quickly down the paper, and that the rates of molecules of different solutes would tend to become more nearly equal. The solute molecules would, because of the mass-action effect, have a decreasing affinity for incorporation in the cellulose-water complex as the number of water molecules in the solvent increased and thus have a greater tendency to remain in the mobile phase and move with it. This, in fact, is found to occur (Fig. 1). It is significant that even at very high water contents (80–90 %) the amino-acids, although moving at almost identical rates, do not lie along the solvent boundary, i.e. their R_F values are very nearly, but not quite, equal to unity. It would appear that at these levels there is still some incorporation of solute molecules in the cellulose-water complex.

In the case of the three water-miscible alcohols which we have studied, the increase in hydrophilic character in the series *n*-propanol-ethanol-methanol would be expected to result in progressively greater competition by the alcohol molecules for incorporation in the cellulose-water complex to the exclusion of solute molecules which consequently would have a greater tendency to move with the mobile phase. This effect is apparent from the general increase of R_F values of the amino-acids through this series of alcohols (Fig. 2).

If a mechanism of this type is assumed to be generally operative in paper chromatography then, in the case of solvents only partially miscible with water, the composition of the flowing solvent and of the cellulose-water complex are presumably variable only within fixed limits. The effect will then become almost equivalent to true partition between solvent and water, and R_F values will be expected to correspond with those calculated from the partition coefficients, as found by Consden *et al.* (1944) in the case of *n*-butanol.

SUMMARY

1. The use of water-miscible solvents for the separation of amino-acids by paper chromatography is described.

2. Aqueous mixtures of relatively non-volatile solvents such as furfuryl alcohol and tetrahydrofurfuryl alcohol give small sharp spots by downward flow in the usual way.

3. With more volatile solvents (lower alcohols, acetone, pyridine and tetrahydrofuran) satisfactory results can be obtained by the method of capillary ascent in small closed glass tanks.

4. Aqueous acetone can with advantage replace collidine for use in conjunction with phenol on two-way chromatograms.

5. The bearing of these results on a possible mechanism for paper chromatography is discussed.

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The Distribution of Cholinesterase Types in Mammalian Tissues

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Much work has been carried out in recent years in order to characterize more fully the mammalian cholinesterases, but, with certain exceptions referred to below, this has been largely limited to a study of these enzymes in brain, red blood cells and plasma. This work has amply demonstrated that fundamental differences in specificity and in reaction kinetics exist between the cholinesterases present in these three tissues (Alles & Hawes, 1940; Glick, 1941; Richter & Croft, 1942; Bissegger & Zeller, 1943), and Mendel and his colleagues have described 'true' and 'pseudo' cholinesterases which hydrolyse respectively acetyl-β-methylcholine and benzoylcholine (Mendel & Rudney, 1943; Mendel, Mundell & Rudney, 1943).

Selective inhibition of these different types of cholinesterase has been described for a number of substances: Hawkins & Gunter (1946) reported inhibition of the pseudo-cholinesterase by the dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)-trimethylammonium bromide (Nu 683). A more recent prostigmine analogue, the *N*-*p*-chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide (Nu 1250), inhibits the 'true' enzyme (Hawkins & Mendel, 1949). Di-*iso*propyl fluorophosphonate (DFP) has also been shown to be a selective inhibitor of the pseudo-cholinesterase (Mazur & Bodansky, 1946; Mendel & Hawkins, 1947), while $\beta\beta'$-dichlorodiethyl-*N*-methylamine hydrochloride (DDM) selectively inhibits the true cholinesterase (Thompson, 1947; Adams & Thompson, 1948). The degree of inhibition of these cholinesterase types has also been studied for certain of the polyphosphate esters, notably hexaethyl tetraphosphate and tetraethyl pyrophosphate (Brauer, 1948; Dubois & Mangun, 1947).

Turning to *in vivo* work, attempts have been made to correlate the signs of toxicity produced by injection of DFP with cholinesterase levels in the blood and in the brain (Mazur & Bodansky, 1946; Freedman, Willis & Himwich, 1949). In view of the early signs of intoxication by this compound (salivation, lachrymation, diarrhoea and muscular twitchings), it would be of interest to know more about the interaction of DFP and other cholinesterase inhibitors with the enzymes present in tissues other than brain and blood. In the first place it is desirable to know whether the two types of cholinesterase described in serum and red blood cells respectively are present also in other tissues, and if so in what relative amounts, or whether further types of cholinesterase with different kinetic and other properties are present in these sites.

Information concerning this distribution is scanty. Mendel & Mundell (1943) have described a pseudo-cholinesterase prepared from dog pancreas, while Langemann (1944), using (1) the influence of substrate concentration on hydrolysis rate, and (2) degree of inhibition by percaine, caffeine and papaverine as his criteria for differentiation, has concluded that human brain and skeletal muscle contain only the true enzyme, while human ovary contains only a pseudo-cholinesterase. Sawyer & Hollinshead (1945) showed that cat peripheral nerve fibres and ganglia contain both true and pseudo-cholinesterases in approximately equal concentrations; they also found that after section and degeneration of the preganglionic fibres the true cholinesterase of the ganglion and of the degenerating preganglionic fibres is lost at a more rapid rate than the pseudo-cholinesterase, a fact which they interpret as minimizing the physiological significance of the latter enzyme in synaptic transmission.

Plattner & Hinter (1930), Gilman, Carlson & Goodman (1939) and others have studied the total cholinesterase activity of a wide range of animal tissues, but no attempt to compare the cholinesterase types in a series of different tissues, using the more recently developed methods of differentiation, was made until the work of Sawyer & Everett (1947). These workers briefly reported on the relative rates of hydrolysis of acetyl-β-methylcholine and benzoylcholine by a number of rat tissues, and showed that liver and certain glandular tissues hydrolyse benzoylcholine more rapidly than acetyl-β-methylcholine; they did not, however, report on the use of any differential inhibitors in these various sites, nor on the cholinesterase types in unstriated muscle, apart from uterus.

The experiments described below represent the initial stages of a more extended comparison of the types of cholinesterase activity of different mammalian tissues. In view of the differences in blood esterases which exist among animals of different species, we have studied, so far, only the tissues of the rat and, in a few instances, of man. We have compared the relative rates of hydrolysis of acetylcholine and of Mendel's 'specific' substrates, acetyl-

β-methylcholine and benzoylcholine, by various rat tissues, and have studied the effect of mixed substrates on these esterases; we have also examined the sensitivity of the cholinesterases of these different tissues to inhibition by DFP.

A preliminary report on this work was given to the Biochemical Society on 16 December 1949 (Ord & Thompson, 1950).

EXPERIMENTAL

Materials. White rats weighing from 100 to 200 g. were used. On account of the sex differences, which have previously been noted by other workers in the cholinesterase levels of various tissues (Birkhäuser & Zeller, 1940; Beveridge & Lucas, 1941; Sawyer & Everett, 1947), only male animals have so far been examined. They were killed by decapitation and exsanguination, after which the carcasses were perfused with 0·9% NaCl introduced through the inferior vena cava and the descending aorta; perfusion was continued until the effluent fluid was no longer visibly blood stained.

The tissues under investigation were then removed, weighed, minced with scissors and finally homogenized in 0·025M-$NaHCO_3$ in a high-speed homogenizer of the type described by Folley & Watson (1948). The dilution of the tissue sample in the final homogenate varied according to the activity of the tissue under examination.

The following tissues have been studied: brain, skeletal muscle (diaphragm, quadriceps femoris), suprarenal gland, stomach, liver, lung, heart (auricle and ventricle), salivary gland (submaxillary), Harderian gland, intestinal mucosa and muscle, and skin. In preparing the intestinal mucosa and muscle the ileum was removed, opened longitudinally, washed free from food debris with 0·9% NaCl, and the mucosa scraped from the muscle layer with a sharp scalpel. Skin was taken from young rats weighing from 8 to 10 g., and was prepared and sliced by the method described by Thompson & Whittaker (1944).

Human tissues were obtained either post-mortem or from material removed by surgical operation. Adherent blood was removed by blotting with filter paper, after which the tissue was finely minced with scissors and washed repeatedly in 0·9% NaCl before homogenizing in 0·025M-$NaHCO_3$.

Estimation of esterase activity. Ammon's (1933) adaptation of the Warburg manometric technique was used. All measurements were made in duplicate at 38° and at pH 7·4, and were corrected for non-enzymic hydrolysis of the substrate. Esterase activity, measured over 0–30 min., is expressed as μl. of CO_2/g. of tissue (wet wt.)/hr., except where otherwise stated.

Substrates. (1) Acetylcholine chloride (ACh) (British Drug Houses, Ltd.); (2) Acetyl-β-methylcholine chloride (MCh) (Savory and Moore, Ltd.); (3) Benzoylcholine chloride (BCh), prepared by Dr A. H. Ford-Moore, Experimental Station, Porton. Each substrate was dissolved in 0·025M-$NaHCO_3$ immediately before use, and a sufficient volume of the solution added to the reaction mixture to bring the final concentration to 0·015M for ACh and BCh, and 0·03M for MCh. Rough determinations of the Michaelis curves have shown that these concentrations lie upon the flat part of the curves obtained with the pseudo-cholinesterase, or are in excess of the peak concentrations for the true enzyme Successive 10 min. readings over the first 30 min. showed either a steady rate of CO_2 production, or at most only a very slight falling off.

Inhibitor. Di-*iso*propyl fluorophosphonate (DFP) was kindly provided by the Experimental Station, Porton. This was dissolved in 0·025M-$NaHCO_3$.

RESULTS

The relative esterase activities of homogenates of different tissues from adult male rats towards ACh, BCh and MCh are shown in Table 1. This table includes the results of at least two experiments on each tissue, and the individual figures across each line refer to a direct comparison of the three substrates in a single experiment. With each tissue further experiments have been done using only a single substrate. The levels of ACh hydrolysis for a given tissue obtained from different rats have in general agreed well; in the case of ventricle, for example, twelve separate experiments on different animals gave a mean value of 3630 μl. of carbon dioxide/g./hr., with a standard deviation of 515. As would be expected, a greater degree of variation has been found among the results obtained with BCh and MCh for those tissues showing only slight activity towards these substrates.

We have confirmed the wide variations in the rates of hydrolysis of ACh by different tissues, a finding which is to be expected when the differences in innervation and cellular make-up are considered. Particularly high values have been found for heart auricle; intestinal mucosa and muscle, Harderian gland and heart ventricle have also shown high activity. In this connexion it is of interest that Antopol, Glaubach & Glick (1939) have shown that the total cholinesterase content of the auricles of rabbit heart is also considerably greater than that of the ventricles.

It will be seen that the relative rates of hydrolysis of BCh and MCh by these various tissues also differ strikingly. For comparative purposes the rates of hydrolysis of these 'specific' substrates are also expressed in Table 1 as percentages of the rate of hydrolysis of ACh by the given tissue. When expressed on this basis it will be noted that the tissues studied appear to fall into three groups: group *A*, tissues which hydrolyse MCh very much more rapidly than BCh, e.g. brain, skeletal muscle and suprarenal gland; group *B*, tissues which hydrolyse MCh and BCh at approximately equal rates, e.g. stomach, liver, lung and salivary gland; group *C*, tissues which hydrolyse BCh very much more rapidly than MCh, e.g. heart ventricle and auricle, intestinal muscle and mucosa, Harderian gland and skin.

The mean MCh/BCh ratio for these three groups is >10, 0·9 and 0·25 respectively. Rat serum, which has been shown by Mundell (1944) to contain both true and pseudo-cholinesterases, has a MCh/BCh ratio of 2·1.

Table 1. *Relative cholinesterase activities of rat tissues towards ACh,† BCh and MCh*

Tissue	Activity (μl. of CO_2/g./hr.) ACh		Activity (μl. of CO_2/g./hr.) BCh		Activity (μl. of CO_2/g./hr.) MCh		Activity as percentage of ACh activity BCh	Activity as percentage of ACh activity MCh	Ratio MCh/BCh
Brain	5250, 5570, 5880	5535	455, 236, 346	346	4040, 4240, 4453	4245	6	75	12
Quad. femoris	114, 245, 188, 376	231	0, 21, 28, 17	16	89, 151, 121, 269	158	7	69	10
Diaphragm	728, 528	628	0, 0	0	334, 350	342	0	54	>54
Suprarenal gland	3180, 1900	2540	93, 0	47	1240, 747	994	2	39	20
Stomach	2060, 1010	1535	467, 267	367	462, 155	309	24	20	0·8
Liver	706, 925, 1105	912	184, 198, 253	212	219, 140, 200	186	23	20	0·9
Lung	992, 703	848	228, 202	215	174, 176	175	25	21	0·8
Salivary gland	1890, 1288	1589	609, 369	489	656, 391	523	31	33	1·1
Heart ventricle*	3300, 3900	3600	1105, 995	1050	315, 300	308	29	9	0·3
Heart auricle*	11600, 9960	10780	2880, 3920	3400	1040, 820	930	32	9	0·3
Intestinal muscle*	3840, 3500	3670	1620, 1690, 1240	1517	590, 685, 571	615	41	17	0·4
Intestinal mucosa*	5320, 7500	6460	3130, 3380	3255	468, —	468	50	7	0·1
Harderian gland	6060, 6360	6210	3180, 3320	3250	515, 288	401	52	6	0·1
Skin*	3500, 2560	3030	830, 694	762	358, 230	294	25	9	0·4
Serum	950, 842	896	136, 88	112	308, 162	235	12	26	2·1

* Activity measured over 0–15 min.
† Acetylcholine chloride, ACh; benzoylcholine chloride, BCh; acetyl-β-methylcholine chloride, MCh.

These findings are consistent with one of two possibilities: first, these three groups of tissues might each contain a characteristic cholinesterase capable of hydrolysing both BCh and MCh in addition to ACh, although at different rates and therefore each showing a different MCh/BCh ratio; such an esterase, by hydrolysing both 'specific' substrates, would differ fundamentally from those studied in brain, red cells, plasma and pancreas. An alternative possibility is that the tissues of the three groups each contain varying amounts of two different enzymes, both of which can hydrolyse ACh, while one can hydrolyse BCh but not MCh, and the other MCh but not BCh, i.e. that the groups each contain varying amounts of the true and pseudo-cholinesterases, in Mendel's sense.

Summation experiments with mixed substrates were therefore carried out on tissues from groups *B* and *C*. From Table 2 it will be seen that for each of the tissues studied the carbon dioxide output in the presence of both substrates amounts to 81–102 % of the sum of the carbon dioxide outputs in the presence of each substrate separately. Since the concentration of each substrate was adequate for maximum rate of enzymic activity over the period studied, the summation occurring in the presence of both substrates suggests that two enzymes are concerned, one hydrolysing BCh exclusively or almost exclusively, and the other MCh.

In order that the rate of simultaneous hydrolysis of two substrates by two related enzymes should equal the sum of the rates of hydrolysis of each sub-

strate separately, it must be assumed that the two enzyme systems function independently of each other, and that neither substrate inhibits the activity of the other enzyme. Butyrylcholine, which has been shown to be a substrate for pseudo-cholinesterase (Stedman & Stedman, 1935; Nachmansohn & Rothenberg, 1945), has been found to act as a competitive inhibitor of the true enzyme (Cohen, Kalsbeck & Warringa, 1949), and Aldridge (1949) has stated that benzoylcholine shows a similar inhibitory action on the true cholinesterase in washed red cells of goat blood, although the action is only about one-third as great as that exerted by butyrylcholine. Although inhibitory effects may account for the finding that only 80–90 % of the arithmetical sum of the rates of hydrolysis of the separate esters was found for some of the tissues studied, such effects clearly do not interfere with the demonstration that complete or almost complete summation of activity occurs with these two substrates under our conditions.

substrate was tipped in was maintained constant, and amounted to 15 min. at room temperature followed by 10 min. in the thermostat while reaching temperature equilibrium. The values given in Table 3 are derived from at least three experiments on any given tissue for a given substrate. From these results an estimate has been made of the concentration of DFP (I_{50} concentration) required to produce 50 % inhibition of esterase activity under standardized conditions *in vitro* (Table 4).

It will be seen from Table 4 that the hydrolysis of BCh by the tissues of group *B*, and to a lesser extent by those of group *C*, is inhibited by very much lower concentrations of DFP than those required to produce an equal degree of inhibition of the hydrolysis of MCh. It must be stressed that for any given tissue the hydrolysis of the various substrates was carried out under identical conditions, so that in each case the system contained similar amounts of inert protein which might theoretically combine with DFP and so reduce the effective concentrations available

Table 2. *Hydrolysis rates of mixed substrates by rat tissues*

	Hydrolysis rate (μl. CO_2/g./hr.)				
			(MCh + BCh)		
	MCh (0·03 M)	BCh (0·015 M)	Found	Calc.	'Summation' (%)
Salivary gland	336	340	616	676	91
Liver	214	226	369	440	84
Lung	199	154	291	353	83
Stomach	214	200	360	414	87
Ventricle	256	1510	1795	1766	102
Intestinal mucosa	1360	5540	6900	6900	100
Intestinal muscle	1050	1920	2400	2970	81

An attempt was also made to carry out and interpret summation experiments using ACh + BCh and ACh + MCh. In every case the rate obtained with either pair of substrates was greater than that obtained with the specific substrate alone, but less than with ACh alone, i.e. no evidence of summation was observed. The results suggested therefore that each of the specific substrates is hydrolysed by an enzyme which also hydrolyses ACh, but owing to the difficulty in reaching an accurate interpretation these experiments were not pursued.

Since, as mentioned above, it has been shown by earlier workers that DFP is a selective inhibitor of the pseudo-cholinesterase of human plasma, experiments have been carried out with this compound in the hope of obtaining further information on the nature of the enzymic hydrolysis of MCh and BCh in these various tissues. The percentage inhibitions of enzymic activity towards ACh, BCh and MCh caused by the addition *in vitro* of varying concentrations of DFP to the various tissue homogenates are shown in Table 3. In these experiments the time of incubation of the homogenate with the inhibitor before the

for producing inhibitory effects. If the hydrolysis of BCh and MCh is carried out by one enzyme it is unlikely that there would be this degree of disparity in sensitivity to DFP by one and the same enzyme preparation. The findings suggest rather that the hydrolysis of BCh by tissues of groups *B* and *C* is brought about by an enzyme of the 'pseudo' type, highly sensitive to inhibition by DFP, whereas the hydrolysis of MCh by these tissues is brought about by a different, relatively DFP-insensitive enzyme, of the type found also in brain and skeletal muscle.

It is of interest that for the groups *B* and *C* preparations the I_{50} value for the hydrolysis of ACh in each instance closely approximates to the value for BCh hydrolysis, even in the case of salivary gland and lung where BCh and MCh are hydrolysed at approximately the same rate (Table 1). Further, when the hydrolysis of ACh is considered it will be noted that salivary gland, lung, ventricle and Harderian gland are all considerably more sensitive than brain or diaphragm to inhibition by DFP. There is also a striking difference in the sensitivity of MCh hydrolysis to DFP, ventricle and Harderian gland being

Table 3. *Inhibition by DFP of the hydrolysis of ACh, BCh and MCh by rat tissues*

(ACh, 0·015 M; BCh, 0·015 M; MCh, 0·03 M. Activity measured over 0–30 min.)

Acetylcholine

Concentration of DFP ($\times 10^{-8}$ M)	0·36	0·54	0·9	1·35	1·8	3·6	4·5	5·4	7·2	9·0	13·5	18·0	45	90	135	180
Tissue	Percentage inhibition															
Brain	—	—	—	—	—	—	—	—	—	30	35	—	57	79	87	94
Diaphragm	—	—	—	—	—	—	—	—	—	47	52	61	74	97	—	—
Lung	27	36	54	—	76	—	—	—	—	—	—	—	—	—	—	—
Salivary gland	22	27	40	—	63	—	80	—	—	—	—	—	—	—	—	—
Ventricle	—	—	32	50	67	83	91	96	98	100	—	—	—	—	—	—
Harderian gland	—	—	17	20	33	43	—	73	—	94	—	—	—	—	—	—

Benzoylcholine

Concentration of DFP ($\times 10^{-8}$ M)	0·18	0·36	0·54	0·9	1·35	1·8	3·6	4·5	5·4	7·2	9·0
Tissue	Percentage inhibition										
Lung	—	15	26	42	58	69	—	92	—	—	—
Salivary gland	24	30	—	55	66	71	—	92	—	—	—
Ventricle	—	—	—	32	50	69	—	95	99	—	99
Harderian gland	—	—	—	20	33	46	—	77	—	—	98

Acetyl-β-methylcholine

Concentration of DFP ($\times 10^{-8}$ M)	1·8	3·6	4·5	5·4	7·2	9·0	13·5	18·0	45	54	72	90	135	180
Tissue	Percentage inhibition													
Brain	—	—	—	—	—	23	36	—	50	—	—	76	93	95
Diaphragm	—	—	—	—	—	21	32	44	63	—	—	95	—	—
Lung	—	—	—	—	—	—	25	33	—	49	67	76	—	—
Salivary gland	—	—	—	—	—	21	28	35	53	—	61	—	—	—
Ventricle	32	42	—	52	—	68	—	—	—	—	—	—	—	—
Harderian gland	12	23	—	39	50	60	—	—	—	—	—	—	—	—

Table 4. *Concentration of DFP required to produce 50 % inhibition of esterase (I_{50})*

(Concentration of substrates: ACh, 0·015 M; BCh, 0·015 M; MCh, 0·03 M)

Group	Tissue	Wt. of tissue (mg./bottle)	I_{50} concentration ($\times 10^{-8}$ M) ACh	BCh	MCh
A	Brain	30	30·0	—	37·0
	Diaphragm	150	12·0	—	27·0
B	Salivary gland	150	1·3	0·8	42·0
	Lung	500	0·9	1·1	42·0
C	Ventricle	210	1·4	1·4	5·0
	Harderian gland	100	3·5	2·2	7·1

very much more sensitive than the tissues of groups *A* and *B*. While it is essential to exercise caution in drawing conclusions from the comparative effects of a single inhibitor on preparations from different tissues, it should be noted that in the case of ventricle the bottles actually contained a greater weight of tissue than in the case of salivary gland, and yet the I_{50} concentration for MCh hydrolysis by ventricle is only one-eighth that for lung, while brain, with only 30 mg. present in each bottle, again falls into the more insensitive group. On present evidence, however, we do not feel justified in concluding that these results indicate that ventricle and Harderian gland contain a different esterase for the hydrolysis of MCh from that possessed by the tissues of groups *A* and *B*.

DISCUSSION

In addition to Sawyer & Everett's (1947) demonstration of the ability of glandular tissues and of uterus to hydrolyse BCh, Augustinsson (1948) has reported that guinea pig small intestine shows approximately equal activity towards both MCh and BCh, while McNaughton & Zeller (1949) have stated that guinea pig parotid gland is a typical source of the pseudo-cholinesterase. The presence of a high BCh-

hydrolysing activity in heart muscle, however, does not appear to have been demonstrated before; indeed Nachmansohn & Rothenberg (1945) have claimed that benzoylcholine is not hydrolysed by the esterase present in the apex of ox-heart muscle. Although we have not studied the apex alone, we have demonstrated a very high rate of hydrolysis of BCh by both rat ventricle and auricle.

When the relative cholinesterase activities of different tissues towards ACh, BCh and MCh are compared (Table 1) one striking fact emerges, namely, that by contrast with brain, skeletal muscle and suprarenal gland, those tissues which we have studied and which receive only post-ganglionic autonomic innervation exhibit an activity towards BCh either equalling or exceeding that towards MCh. From this limited survey of different tissues it would seem that in the case of the rat those tissues in which acetylcholine exerts a nicotine-like action (i.e. in which it is concerned with transmission to another neurone or to a striated muscle cell), contain predominantly the true cholinesterase, while those in which it exerts a muscarine-like action contain both true and pseudo-cholinesterases in such amounts that the hydrolysis rate of BCh either equals or exceeds that of MCh.

The inclusion of brain as a tissue in which acetylcholine exerts a nicotine-like action may be questioned on the grounds that many of the central actions of acetylcholine are sensitive to atropine. Feldberg (1945), however, has pointed out that atropine sensitivity should not be regarded as the sole test for differentiation of the actions of acetylcholine, and concludes that its central effects should be considered together with its actions on peripheral ganglia and motor end-plates.

The question at once arises as to whether the pseudo-cholinesterase in these tissues is concerned physiologically with transmission processes, or with some other metabolic function unconnected with acetylcholine and conduction, and only brought into prominence when the cell structure is disorganized in homogenized preparations. The evidence we have at our disposal is at present inadequate to answer this question. In connexion with the physiological roles of these tissue esterases it is worth recalling that acetyl-β-methylcholine exhibits mainly muscarine-like actions, a fact which may be relevant to our finding that this compound is more rapidly hydrolysed by 'nicotinic' than by 'muscarinic' tissues. Further, butyryl choline has been found by Adams (1949) and Adams & Whittaker (1949) to be hydrolysed rapidly by the pseudo-cholinesterase of plasma, but hardly at all by the true cholinesterase of erythrocytes, and Chang & Gaddum (1933) showed that while butyrylcholine had only negligible pharmacological activity on rabbit intestine and on rabbit blood pressure it was highly active on the frog rectus abdominis and in eserinized leech muscle preparations. These observations suggest, therefore, that butyrylcholine exhibits mainly a nicotine-like action, which would accord with our finding of high pseudo-cholinesterase activities in the 'muscarinic' sites. If it is legitimate to relate the types of action of these choline esters to the relative amounts of the two types of esterase present in different tissues, it would suggest that the pseudo-cholinesterase in these autonomically innervated tissues is situated, even in intact preparations, in such a way as to be able to inactivate circulating butyrylcholine, and not intracellularly in such a way as to be inaccessible to circulating substances or to substances released at or near the cell surface.

By contrast with this wide distribution of the pseudo-cholinesterase in the rat, it has been claimed by Gunter (1946) that in the ox and sheep a number of glandular tissues are unable to hydrolyse benzoylcholine, but no other properties were examined to determine whether any further differences could be detected among the various tissue esterases present in these species. The observations of Sawyer & Everett (1947) and of ourselves in rats, may, however, be of significance in human physiology and pathology, since in preliminary experiments with human tissues we have found an active pseudo-cholinesterase in certain areas of the brain and also in uterus, ureter and jejunal muscle and mucosa.

SUMMARY

1. A survey has been made of the relative rates of hydrolysis of acetylcholine, benzoylcholine and acetyl-β-methylcholine by various different rat tissues.

2. It has been found that benzoylcholine and acetyl-β-methylcholine are hydrolysed at approximately equal rates by stomach, liver, lung and submaxillary gland, while in the case of heart auricle and ventricle, intestinal muscle and mucosa, Harderian gland and skin, benzoylcholine is hydrolysed more rapidly than acetyl-β-methylcholine.

3. Summation experiments, and observations on the degrees of inhibition produced by di-*iso*propyl fluorophosphonate (DFP), indicate that these tissues contain two cholinesterases, one DFP-sensitive and the other relatively DFP-insensitive, i.e. that they contain both true and pseudo-cholinesterases in Mendel's sense.

4. With acetylcholine as substrate, salivary gland, lung, ventricle and Harderian gland are much more sensitive than brain or skeletal muscle to *in vitro* inhibition by DFP.

5. The possible significance of these results in connexion with the nicotine-like and muscarine-like actions of choline esters and with the effects produced by cholinesterase inhibitors is discussed.

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The Virulence-enhancing Factor of Mucins

1. A BIOLOGICAL ASSAY OF VIRULENCE-ENHANCING ACTIVITY

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Nungester, Wolf & Jourdonais (1932) discovered that a marked increase in the virulence for mice of strains of *Staphylococcus aureus*, *Streptococcus pneumoniae* (Type II) and *Strep. haemolyticus* could be effected by injecting intraperitoneally a suspension of the organisms in 5% (w/v) hog gastric mucin. About the same time Miller (1933) found that the same procedure would reduce the minimum lethal dose for mice of strains of *Neisseria meningitidis* by 10^{-6}.

Since that time the virulence-enhancing power of mucin (review: Olitzki, 1948) has been used extensively, particularly in serum and chemotherapeutic testing, for the production of experimental infection in several different test animals; relatively small numbers of bacteria of many different species have been used. The intratracheal route of infection for the production of pneumonias has been used with a success equal to that of the more widely exploited intraperitoneal route. Most workers have used dried commercial hog gastric mucin, mainly but not solely the 'Granular Mucin 1701 W' of Wilson Laboratories, Chicago, U.S.A. Anderson & Oag (1939) and Tunnicliffe (1940) are in agreement on the activity of

human salivary mucin; human gastric mucin (Sindbjerg-Hansen, 1943), hog intestinal mucin (Levaditi & Vaisman, 1937) and guinea-pig intestinal mucin (Cantacuzène & Marie, 1919) have also been reported active, but these observations have yet to be confirmed. It must be pointed out that mucin enhances the virulence of pathogenic or potentially pathogenic (e.g. *Escherichia coli*) organisms, but has no effect on non-pathogens such as *Bacillus subtilis* and Sarcinae.

The method by which pathogenic bacteria attack intact epithelial surfaces and eventually cross them in order to invade other tissues is almost unknown. Pronounced specific antibacterial action of mucin lining the alimentary tract, due to the presence of lysozyme, has been reported to be absent by Goldsworth & Florey (1930); these authors suggest that any protective function against bacteria lies in the mechanical properties of the mucin. However, the well-established phenomenon described above suggests that mucins coating these epithelial surfaces may well help bacteria in their initial attack. Hill, Huffer & Nell (1945) have obtained more direct evidence on this point. They found that strains of *Neisseria intracellularis*, when mixed with mucin, survived far more easily on the mouse vaginal surface than when mixed with saline.

Thus the investigation of mucins for a chemical entity producing the virulence-enhancing effect is an interesting problem, and papers in this series will describe researches to this end.

An essential prerequisite was the development of a statistically adequate method of biological assay. This necessarily involves large-scale animal work, but facilities are available for such work and this communication describes the successful establishment of the assay. In essence it consists of a comparison of unknown mucin fractions with a 'standard' mucin preparation. A constant number of *Bacterium typhosum* (*Salmonella typhosa*), which is far below the normal lethal dose, is injected intraperitoneally into mice in the presence of falling concentrations of the two mucins. Comparisons of death rates and mucin concentrations are made using probit analysis.

EXPERIMENTAL

The 'standard' mucin. A large quantity (10 lb.) of one batch of 'Granular Mucin 1701 W' of Wilson Laboratories, Chicago, U.S.A., was finely powdered in a drug disintegrator, passed through a No. 80 sieve and thoroughly homogenized by rolling in a ball mill for 2 days. It is kept over P_2O_5 and NaOH at 0° and contains 6% moisture (loss at 0·1 mm. Hg and 60° over P_2O_5). The density of this mucin was found to be approximately unity. A 1·75% (w/v) suspension needed for the assay is made by adding 0·93 g. of the solid to 49·1 ml. of sterile water containing enough 0·1 N-NaOH to give a final pH 7·0–7·2. The mixture is homogenized in a special homogenizer described by Folley & Watson (1948). If the homogenizer is not used the mucin is difficult to disperse efficiently.

Unknown mucin samples. The densities are taken as unity. Suitable dilutions for injection are made by adding a quantity of sample (calculated with reference to the substance dried at 0·1 mm. Hg at 60° over P_2O_5) to the necessary quantity of sterile water containing sufficient 0·1 N-NaOH to give a final pH 7·0–7·2. The mixture is homogenized in the special homogenizer, or if foaming is excessive, in a bottle with glass beads.

The killing of vegetative organisms in mucin suspensions. Filtration of the mucin samples is impossible because a large part of the material remains in suspension. Autoclaving definitely reduces the virulence-enhancing activity (see Discussion), and produces variable amounts of deposit. To avoid the effects of excessive heat on the virulence-enhancing factor, the mucins are placed in a water bath at 60° for exactly 0·75 hr. in order to kill vegetative pathogens. The degree of contamination with heat-resistant organisms is noted by plating out 0·1 ml. samples of all 'standard' and unknown mucins on tryptic meat agar, incubating aerobically and anerobically, and counting and examining the colonies. Thus prepared, the 'standard' mucin, contains about thirty sporing organisms per injection dose (0·45 ml.). The same result has been obtained with a very large number of mucin fractions, which, during their chemical preparation, have been filtered through a bacterial candle when in solution in a suitable solvent. In some cases, however, the unknown mucin fractions were precipitates, and at this stage of the fractionation, filtration was not possible; such mucin samples had a greater contamination with Gram-positive rods. In all the bioassay tests, groups of control mice received mucin only, and there is no evidence that any of the organisms present in the samples are pathogenic.

The test organism. The 'Ty_2' strain of *Bact. typhosum* was selected as the test organism. This strain, originally isolated in 1918, is the classical strain of maximum virulence and O inagglutinability, widely employed as a vaccine strain and for the preparation of the 'test dose' in mouse-protection tests (Felix, 1938; private communication). It was used at the Lister Institute of Preventive Medicine, London, by Henderson (1939). The organism was kept at low temperatures in dried gelatine-ascorbic acid culture as described by Stamp (1947). A fresh tube of dried culture is used every week; three progressive subcultures are made during the week for three assays. The dried culture is dissolved in a small amount of warm tryptic meat broth and spread on four tryptic meat agar slopes which are incubated overnight at 37°. At the same time a tryptic meat agar plate is streaked to make certain the colonies are uniformly smooth. The four slopes are washed off with tryptic meat broth, diluted to opacity no. 2 (Brown's tubes) equivalent to 10^9 organisms/ml. Tenfold dilutions in tryptic meat broth are made to 10^5 organisms/ml. and the dilutions stored for a short time in ice water ready for mixing with the mucin. The mixing is done immediately prior to injection. A viable count is made by the method of Miles, Misra & Irwin (1938) on the 10^5/ml. dilution. This method consists of placing drops of known volume of a suitable dilution on a tryptic meat agar plate and spreading by rotation. The plates are incubated overnight, and colonies (roughly 1000) are counted. Since a constant number of organisms are needed for the assay, on the very few occasions when this count is not within the limits $0·8–1·2 \times 10^5$ organisms/ml. the assay is repeated.

The test animal. A strain of white mouse is bred for the Microbiological Research Department by the Ministry of Supply Animal Farm. Weight limits are 18–20 g.

The route of infection. Each mixture (0·5 ml.) is injected intraperitoneally.

The form of the assay. The composition of the injection solutions and the form of the assay is shown in Table 1.

The dilutions x and y% of the unknown mucin fraction are obtained from a preliminary test in which more and wider mucin dilutions are injected with 5×10^3 organisms into batches of ten mice. Mucin and organism controls, together with the 'standard' mucin, are omitted from this preliminary test. Occasionally, if little material is available, only one dilution of the unknown mucin is used.

To avoid inaccuracy in pipetting viscous mucins they are added to the suspensions of organisms from a pipette calibrated to contain; the mixing is then done with this same pipette.

The duration of observations. The majority of deaths occur on the second and third days, but the animals are kept until the fifth day after injection.

Deaths of mucin controls. Occasional deaths in mucin controls are disregarded, but should the death rate be more than two in a batch of twenty mice, then the assay is repeated using lower mucin dilutions.

Table 1. *Form of the virulence-enhancing assay*

Injection solution (0·5 ml./mouse)		No. of *Bact. typhosum* (Ty_2) per injection	No. of mice	Expected death rate (%)
Nature	Composition			
Organism control	9 vol. tryptic meat broth 1 vol. 1×10^7 dilution Ty_2	5×10^5	10	Nil
Organism control	9 vol. tryptic meat broth 1 vol 1×10^8 dilution Ty_2	5×10^6	10	20–80
Standard mucin 1·75% (w/v) control	9 vol. mucin 1·75% (w/v) 1 vol. tryptic meat broth	Nil	10	Nil
Standard mucin 1·75% (w/v) +organisms	9 vol. mucin 1·75% (w/v) 1 vol. 1×10^5 dilution Ty_2	5000	20	50–75
Standard mucin 1·25% (w/v) +organisms	9 vol. mucin 1·25% (w/v) 1 vol. 1×10^5 dilution Ty_2	5000	20	25–50
Unknown mucin x% (w/v) control	9 vol. mucin x% (w/v) 1 vol. tryptic meat broth	Nil	20	Nil
Unknown mucin x% (w/v) +organisms	9 vol. mucin x% (w/v) 1 vol. 1×10^5 dilution Ty_2	5000	20	50–75
Unknown mucin y% (w/v) +organisms	9 vol. mucin y% (w/v) 1 vol. 1×10^5 dilution Ty_2	5000	20	25–50

RESULTS

Comparison of the unknown mucin with the 'standard' mucin preparation is made using probit analysis. The slope of the regression of probit mortality on log dose, necessary for this analysis of one particular assay, is obtained from a regression line constructed from twelve observations on the 'standard' mucin, made over the current period of roughly 3 weeks. This regression line is reassessed every 3 weeks, and therefore takes into account seasonal variations of mice, etc.

The virulence-enhancing strength of the unknown mucin in terms of the 'standard' mucin preparation is expressed in units which are arbitrarily called 'Wilson units'.

Over 100 different unknown mucin samples have been assayed. Table 2 shows the results of duplicate assays on a few samples obtained at important stages in the chemical fractionation of hog gastric mucin (see following paper). The fiducial limits shown in the table are representative of those obtained for other mucin fractions which were only assayed once. Weighted means are included, together with their fiducial limits, showing the increased accuracy obtained by duplicate assays.

DISCUSSION

In establishing a biological assay for virulence-enhancing activity, the effect of two possible variables that could not be eliminated was minimized. These were small changes in inherent virulence that might occur in the chosen test organisms, and also the inaccuracy inherent in counting viable bacteria. For these reasons all unknown mucin samples were compared with a 'standard' mucin preparation in the presence of the same amount of the same dilution of the test organism.

Bact. typhosum (strain Ty_2) was chosen as the test organism, because of its low pathogenicity for mice without mucin. Also it would probably maintain its inherent virulence at a reasonably constant level, provided conditions of growth and dilution were kept standard. Nevertheless, controls which would detect gross changes in the inherent virulence were included in every assay. These controls in fifty or so assays show that the *Bact. typhosum* was constant in killing

two to eight out of ten mice with 5×10^6 organisms, and none, or perhaps an odd one, with 5×10^5 organisms. The workable limits of agreement between the viable count and the initial opacity tube count on which the dilutions for injection are made, are $\pm 20\%$. In view of this fact 5×10^3 organisms were used as the constant test dose with mucin; it was thought that the possible variations on smaller numbers, say 5×10^2 might affect the assay unduly, in spite of the comparison between unknown and standard. The chosen dose, therefore, represents a virulence-enhancing factor of 10^3 if a death point of 50% is obtained with mucin.

The large batches of mice used in this assay are obviously of paramount importance to obtain reasonably accurate results.

sterilize the mucins for vegetative pathogens by standing them for long periods with organic solvents, which were then removed under reduced pressure prior to making the suspensions. The attempts proved unsuccessful and the method described above was instituted. This has yielded excellent results, as evidenced by the constancy of the 'standard' mucin death rates and slopes (5·0–7·5) of the regression lines. Contamination of unknown mucin samples with many sporing organisms is prevented by filtration through a bacterial candle as soon as this is feasible in the fractionation procedure.

It is obvious from Table 2 that agreement between duplicate results is remarkably good for a biological assay of this nature. Consideration of the fiducial limits shows that the assay is capable of detecting a

Table 2. *The results of replicate assays on various mucin fractions together with their weighted means*

Mucin	Assay	Most probable result in 'Wilson units'	Fiducial limits ($P=0.95$) Minimum	Maximum
31	1	1·11	0·93	1·33
	2	0·90	0·64	1·27
	Weighted mean	**1·06**	**0·91**	**1·24**
53	1	1·88	0·97	3·22
	2	1·52	0·70	2·56
	Weighted mean	**1·71**	**1·14**	**2·55**
98	1	2·43	1·51	3·25
	2	2·66	1·77	3·56
	Weighted mean	**2·55**	**1·96**	**3·32**
100	1	1·41	0·80	2·03
	2	1·41	0·93	1·90
	3	1·62	1·28	2·06
	Weighted mean	**1·53**	**1·28**	**1·83**
102	1	6·69	5·19	8·41
	2	5·22	4·04	6·55
	Weighted mean	**5·91**	**5·06**	**6·90**
115	1	1·31	1·06	1·78
	2	1·37	1·10	1·85
	Weighted mean	**1·34**	**1·14**	**1·57**
131	1	3·03	1·95	4·24
	2	2·46	1·44	3·57
	Weighted mean	**2·77**	**2·20**	**3·50**
A	1	0·77	0·63	0·95
	2	0·63	0·50	0·76
	Weighted mean	**0·69**	**0·61**	**0·79**

The method of sterilizing the mucin suspensions presented some difficulty. Filtration was out of the question. Several workers have mentioned that autoclaving at 15 lb./sq.in. for 15–20 min. reduces the activity, and Anderson & Oag (1939) have recorded that two such autoclavings destroy practically all the activity. A quantitative measure of the loss of activity on autoclaving has been ascertained. The 'standard' mucin at pH 7·0–7·2 was autoclaved at 15 lb./sq.in. for 20 min.; the virulence-enhancing activity (1 'Wilson unit') dropped to 0·69 (0·61–0·79) 'Wilson unit'. Attempts were made to

twofold difference in virulence-enhancing power with probability $P=0.95$: somewhat smaller differences can be demonstrated with the same probability in some cases. On the occasions when, to economize in material, only one dilution of the unknown is used, the fiducial limits show a slightly wider range.

The assay therefore fulfilled the requirements for the chemical fractionation of mucins for a virulence-enhancing factor. Its use in the initial part of these fractionations is described in the following paper.

The question of the toxicity of hog gastric mucin has arisen in the course of this large-scale animal

work. Hirooka (1942) reported that hog gastric mucin prepared by alkaline extraction of the mucosa and precipitation with acetic acid was toxic to mice. Samples obtained at all stages in the chemical fractionation described in the following paper were appreciably toxic at concentrations two to three times those which demonstrated virulence enhancement; the same was true of the 'standard' mucin. Mouse post-mortems and the histological examination of various tissues have not revealed the nature of this toxicity, the elucidation of which needs further experimental data.

SUMMARY

1. A biological technique has been established for the assay of the virulence-enhancing factor of mucins and chemical fractions thereof.

2. Unknown mucin samples are compared with a 'standard' mucin preparation. A constant number of *Bacterium typhosum* (strain Ty_2) is injected intraperitoneally into batches of twenty mice in the presence of falling concentrations of the test materials. Comparisons of death rates and concentrations are made by probit analysis.

3. The assay can detect twofold differences in virulence-enhancing activity with probability $P = 0{\cdot}95$.

4. The toxicity of mucin preparations at high concentrations is noted.

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The Virulence-enhancing Factor of Mucins

2. FRACTIONATION STUDIES ON HOG GASTRIC MUCIN

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Almost the whole of the extensive published work on the virulence-enhancing action of mucin deals with the bacteriological study and application of the phenomenon, rather than with the chemical nature of an agent causing the effect. It is evident from this literature, however, that batches of commercial mucin vary greatly in virulence-enhancing power, and for any particular mucin, activity is proportional to the concentration.

A few recorded attempts have been made to isolate a possible virulence-enhancing factor from hog gastric mucin. These attempts which are summarized below suffer from two serious disadvantages. First, the details of a statistically adequate biological assay, capable of a reasonably accurate measurement of virulence-enhancing activity, have never been published. Secondly, all workers have used dried commercial hog gastric mucin, of unknown history.

Anderson & Oag (1939) used the hog gastric mucin prepared by L. Light and Co., London, and as a qualitative test for activity, injected mice intraperitoneally with a strain of *Meningococcus* (*Neisseria meningitidis*). After extracting inactive matter with cold water, an active fraction giving protein and carbohydrate reactions was obtained by extraction with 0·33 N-KOH solution and pre-

cipitation with acetic acid. Hydrolysis of this material with NaOH yielded an active product showing protein reactions only.

Olitzki & Koch (1945) repeated and confirmed the work of Anderson & Oag (1939). Two different samples of mucin were used, and the test for activity involved the intraperitoneal injection of *Shigella dysenteriae* (*Shiga*) into mice. In the case of one mucin (Nordemark-Werke) the material insoluble in alkali was intensely active; it contained 40·5% of inorganic matter, a large proportion of which was aluminium.

Gould & King (1947) fractionated Wilson's 'Granular mucin' by differential centrifugation, and used a test involving the intraperitoneal injection of a strain of Meningococcus into mice; no details of this test were given. Centrifuging an aqueous suspension at 15,000–20,000 rev./min. separated an active precipitate from an inactive viscous supernatant liquor. This precipitate was dissolved in Na_2CO_3 solution and reprecipitated with acetic acid at pH 3. A concentration of twenty times was claimed, and colour reactions showed this active residue to be of a protein rather than carbohydrate nature.

King & MacCabe (1949), after stating that the above method was difficult to repeat, described a slightly different procedure. After high-speed centrifugation of the aqueous suspension, the dried active precipitate was extracted with formamide. Removal of the formamide by dialysis, and freeze drying yielded an active product insoluble in water at pH 7 and giving both protein and carbohydrate reactions. A concentration of twelve times was claimed.

Morgan & King (1943) stated that completely soluble blood-group A substance from Wilson's 'Granular mucin', still retained the virulence-enhancing effect of the original mucin, although heat sterilization destroyed the effect, together with the viscosity of the solution. Electrophoresis showed the preparations to contain 90% of a single component, and 10% of an ill-defined contaminant.

Recently, Landy & Batson (1949) obtained blood-group A substance from Wilson's 'Granular mucin' in 22% yield by the method of Morgan & King (1943), and in 15·6% yield by the method of Zittle (1948). These samples in 3% concentration had approximately the same virulence-enhancing activity for *Bacterium typhosum* (*Salmonella typhosa*) in mice as the original 'Granular mucin' had in 5% concentration.

It is obvious that the chemical fractionation of hog gastric mucin for a possible virulence-enhancing factor is still in the initial stages. The reported activity of totally different mucin fractions is undoubtedly due to inadequate purification.

This paper describes preliminary steps in the chemical fractionation of a virulence-enhancing factor from hog gastric mucin. It has been proved that, although this is the most important factor in virulence enhancement, it is not the only one involved. A smaller, but nevertheless important, part in the enhancement is due to another substance or substances; this has no inherent virulence-enhancing activity, but, when mixed with the virulence-enhancing factor, greatly increases its activity, probably by providing a viscous medium for its dispersion. A brief summary of this work has already been reported (Smith, 1950).

A biological assay capable of detecting a twofold difference in virulence-enhancing activity has been described in the previous paper. This assay was the keystone for the chemical fractionation of hog gastric mucin described in this paper. Fresh hog stomach linings were used as starting material rather than the dried commercial product.

EXPERIMENTAL AND RESULTS

Each fractionation method described below was repeated at least once, using material from a different initial group of hog stomachs. The virulence-enhancing activity of different fractions is given in arbitrary 'Wilson units', with fiducial limits in brackets, for probability $P = 0{\cdot}95$.

Fresh hog stomach linings. Hog stomachs were obtained from C. T. Harris Ltd., Calne, Wilts. On removal from the freshly killed pigs the unwashed stomachs were packed in ice, and within 3–6 hr. were cut open and washed under a slowly running tap; fast jets of water which would remove the adhering mucus were avoided. The inner mucosae were then separated from the outer muscular layers. Should extraction be postponed for a short time, the mucosae were kept at −20°.

Initial extraction of individual hog stomachs

The literature outlined above suggested the use of two initial extraction procedures on individual hog stomachs; the first involved a pepsin autolysis to extract crude blood-group substances (A and H), and the second, an Na_2CO_3 extraction, was used to prevent the protein-splitting action of pepsin.

Crude blood-group substances (A *and* H), *from individual linings.* One minced mucosa was mixed with water (300 ml.), conc. HCl (8 ml.) and toluene (20 ml.), and placed in an incubator at 37° for 1 week; the mixture was shaken at intervals to prevent tissue from rising and rotting on the surface. After the second day the pH was determined daily and maintained at pH 3·0±0·2. The bulk of the tissue dissolved, leaving some insoluble matter; most of the latter was removed by centrifuging in an angle centrifuge and straining the centrifugate through lint. The separation was not complete, and the solution remained slightly turbid. It was cooled to 0° and mixed with absolute industrial ethanol (5 vol.) at −18°. After standing overnight at −18° the white solid was centrifuged off, washed once with industrial ethanol (200 ml.), and dried with absolute industrial ethanol and ether; the ether was removed in a vacuum desiccator over P_2O_5. Yield 3–5 g. One hundred and twenty individual mucosae were extracted in this manner and the samples kept separately.

Extraction of individual hog stomach linings with 0·1 N-*sodium carbonate solution.* One minced mucosa was mixed with 0·1 N-Na_2CO_3 solution (1 l.) in a Waring blender. Toluene (20 ml.) and some glass beads were added and the mixture was shaken at room temperature for 36 hr.; the pH during this shaking was between 9 and 10. Residual tissue was removed by spinning in a Laval centrifuge and straining the centrifugate through lint. The solution was cooled to 0° and adjusted with acetic acid to pH 4·2±0·2, when a precipitate formed. The crude material was precipitated completely by adding absolute industrial ethanol (5 vol.) at −18°. After standing overnight at −18° the pre-

cipitate was washed once with industrial ethanol (200 ml.), and dried as described for the crude blood-group substance. Yield 8–10 g. Seventy-seven individual mucosae were extracted by this method, and the samples kept separately.

Comparison of blood-group activities with virulence-enhancing activities of samples extracted by the above two methods. Both the crude blood-group substance samples and those got by extraction with sodium carbonate possessed virulence-enhancing activity. It was decided to compare virulence-enhancing activities of different samples with their blood-group activities. A parallelism between these activities would have been a good indication that blood-group substances (**A** and **H**) were responsible for virulence enhancement.

The serological examination of all the individual samples extracted by the two methods was kindly carried out by Dr W. T. J. Morgan and Mr E. F. Annison at the Lister Institute of Preventive Medicine, London. Full details of this examination have been published (Annison, Chadwick, Morgan & Smith, 1949); it entailed the comparison of the unknown samples with 'standard' **A** substance and 'standard' **H** substance, in tests involving the inhibition of agglutination of **A** or **O** red blood cells by their respective antisera. Table 1 shows the virulence-enhancing activities of certain samples which have widely differing blood-group activities.

Table 1. *Comparison of blood-group activities and virulence-enhancing activities of the same samples of mucin*

Sample and method of preparation	Blood-group **A** activity (in terms of a standard '**A**' preparation) (%)	Blood-group **H** activity (in terms of a standard '**H**' preparation) (%)	Virulence-enhancing activity ('Wilson units')
Autolysis 1	Nil	200	0·33 (0·25–0·42)
2	Nil	50	0·35 (0·27–0·46)
3	100	3	0·36 (0·27–0·46)
4	25	1·5	0·23 (0·17–0·32)
Sodium carbonate 1	Nil	6	0·39 (0·30–0·51)
2	1·5	Nil	0·29 (0·22–0·38)

It is evident that no parallelism exists between the two activities, and therefore it is unlikely that the virulence-enhancing factor, and blood-group substances (**A** and **H**) are one and the same. In view of this fact, all individual samples of crude blood-group substance were bulked, prior to attempts to separate the virulence-enhancing factor from the blood-group mucoid; the activity of this bulked sample was 0·34 'Wilson unit' (0·26–0·45). Similarly, the sodium carbonate extracts were bulked and had an activity of 0·36 'Wilson unit' (0·28–0·47).

Fractionation of the bulk crude blood-group substance and bulk sodium carbonate extract

Each sample was separated into three main fractions: (1) insoluble at pH 4·2 on prolonged dialysis against distilled water at 0°; (2) soluble at pH 4·2 and precipitated by ethanol; (3) substances passing out of the dialysis bag. From both starting materials the activity was concentrated in the first fraction.

Crude blood-group substance. The crude material (25 g.) was suspended in water (500 ml.) in a Waring blender and the pH adjusted to 4·2±0·2 with 2 N-acetic acid (approx. 12 ml.). The slightly viscous mixture was dialysed against frequent changes of distilled water at 0° for 6 days; the pH remained constant at 4·2±0·2 and a precipitate separated.

The dialysate was adjusted to pH 7·0–7·5, freeze dried, extracted with industrial ethanol (500 ml.) to remove any Na acetate, and finally dried as described for crude blood-group substance. Yield 2·5 g. It was found to be completely inactive.

The contents of the dialysis bag were spun for 1 hr. in the closed bowl of a Sharples centrifuge. The supernatant was filtered through a bacterial candle, cooled to 0° and precipitated with absolute industrial ethanol (5 vol.) at −18°. After leaving overnight at −18° the stringy white solid was washed once by blending with industrial ethanol (1 l.); it was then dried as described for crude blood-group substance. Yield 15 g. From its method of preparation this material, which formed a viscous aqueous solution, contained the blood-group substances (**A** and **H**); this was checked by an inhibition test (Annison, private communication). It had, however, only a trace of virulence-enhancing activity, less than 0·22 'Wilson unit' (0·17–0·29).

The solid remaining in the Sharples bowl was resuspended in water (200 ml.) containing a few drops of 2 N-acetic acid, and recentrifuged. This washing was repeated, and the substance was dried as described for crude blood-group substance. Yield 2·75 g. It was found to have an activity of 1·27 'Wilson units' (0·70–2·05). In a larger scale experiment (300 g. of crude product) this material was not isolated as such, but was separated into two fractions by means of 0·2 N-Na_2CO_3 solution. The solid in the Sharples bowl was stirred with 0·2 N-Na_2CO_3 solution, then spun for 1 hr. in the closed bowl. The supernatant was filtered through a bacterial candle, the pH adjusted to 4·2 with acetic acid, and dialysed for 4 days at 0° against frequent changes of distilled water. The precipitate which formed was spun off, and dried as described for crude blood-group substance. Yield 13·5 g. It was moderately active, 0·43 'Wilson unit' (0·33–0·55). The material insoluble in 0·2 N-Na_2CO_3 solution was washed twice with industrial ethanol and dried as described for crude blood-group substance. Yield 5·0 g. This product was appreciably toxic at 0·3% (w/v), but at 0·2 and 0·15% (w/v) showed virulence-

enhancing activity equivalent to 7·83 'Wilson units' (6·60–9·31).

Sodium carbonate extract. By a similar method the crude material (100 g.) was separated into fractions: (1) Insoluble at pH 4·2; yield 39 g.; activity, 0·42 'Wilson unit' (0·37–0·49). (2) Soluble at pH 4·2 and precipitated by ethanol; yield 15 g.; only a trace of activity, less than 0·20 'Wilson unit' (0·16–0·26). (3) Dialysable material; yield 25 g.; completely inactive.

The direct extraction from fresh stomach linings of material insoluble at pH 4·2*: further purification of this product*

Sodium carbonate extraction was abandoned in favour of autolysis. In view of the sparing solubility of the active material, the substance which remained insoluble after autolysis, and which was to a very large extent discarded in the method of extraction for crude blood-group substances, was retained in the product.

Material insoluble at pH 4·2 *obtained direct from fresh mucosae.* Minced linings from thirty stomachs were autolysed under exactly the same conditions as described for individual mucosae. The whole of the autolysate was strained and precipitated with industrial ethanol (5 vol.). The precipitate was blended with water, brought to pH 4·2 and dialysed at 0° for 1 week. The precipitate was removed and freeze dried. Yield 210 g. A large amount of fat was removed by washing four times with ether (2 l.) and the product was finally dried in a vacuum desiccator over P_2O_5. Yield 100 g. Activity, 0·73 'Wilson unit' (0·46–0·98). The fat recovered from the ether extracts was completely inactive.

Extraction with water at pH 6·0. The above material (100 g.; activity, 0·73 'Wilson unit'), was blended with water (1·5 l.), the pH adjusted to 6 and the mixture left at 0° overnight. The precipitate was spun off in a Laval centrifuge, washed twice at 0° by water at pH 6, and freeze dried. Yield 45 g. Activity, 1·20 'Wilson units' (0·81–1·55). The combined supernatants were filtered through a bacterial candle and freeze dried. Yield 51 g. This product was only very slightly active, 0·30 'Wilson unit' (0·18–0·41).

Extraction with 0·1 N-Na_2CO_3 *solution.* The above product (5 g.; activity, 1·20 'Wilson units') was blended with 0·1 N-Na_2CO_3 solution (100 ml.) and left at 0° overnight; the pH was 10·0–10·5. After spinning at 4000 rev./min. in a refrigerated centrifuge at 0° for 8 hr., the slightly turbid supernatant liquor was removed, the pH adjusted to 6 with acetic acid, and set aside. The solid material was washed a further three times at 0° with 0·1 N-Na_2CO_3 solution (50 ml.) and finally was suspended in water, dialysed at 0° for 2 days to remove Na_2CO_3, and freeze dried. Yield 2·4 g. Activity, 1·82 'Wilson units' (1·01–2·9). The combined supernatants were dialysed at 0° for several days, then freeze dried. Yield 2·1 g. It was moderately active, 0·68 'Wilson unit' (0·38–1·25).

The method finally adopted for the preparation of a reasonably active product direct from hog stomach linings

In spite of the increased activity of the residue after extraction with 0·1 N-Na_2CO_3 solution, this method was precluded from the general procedure by the relatively poor separation effected and the moderate activity of the extract. Non-inflammable dichlorethylene was substituted for ether as a fat solvent.

The whole autolysate, prepared as above, from thirty stomachs (approx. 10 l.) was strained to remove gross particles, and poured into absolute industrial ethanol (40 l.). The precipitate was dried by stirring with absolute industrial ethanol (10 l.) and defatted by stirring three times with dichlorethylene (9 and 2×6 l.). After evaporating the dichlorethylene, the white powder was extracted at 0° overnight four times with water (4·5 and 3×3 l.) at pH $6{\cdot}0 \pm 0{\cdot}2$. The washed precipitate was freeze dried. Yield 25–40 g. The activities of three typical batches of product prepared in this manner were 1·71 'Wilson units' (1·14–2·55), 2·55 'Wilson units' (1·96–3·32) and 2·53 'Wilson units' (2·09–3·08).

Removal of foreign matter. To remove small amounts of foreign matter a large number of solvents were examined for dissolving the active material, which hitherto had been obtained only as a precipitate. Those containing a phenolic group were the most promising, and success was achieved using saturated Na salicylate solution. Before describing the use of this solvent, it is relevant to report the failure of attempts to use formamide as a solvent as described by King & MacCabe (1949). The formamide was purified by repeated recrystallization before use. One of the above mucin fractions (5 g.; activity, 1·2 'Wilson unit') was extracted four times with formamide (50 ml.). The combined formamide solutions were dialysed against frequent changes of distilled water until free of formamide, when a precipitate formed. The suspension in the dialysis bag was then freeze dried. Yield 0·6 g. Activity, 0·85 'Wilson unit' (0·68–2·13).

Use of saturated aqueous sodium salicylate for solution and filtration of the active material, followed by precipitation with ethanol. The crude active material (15 g.), prepared as described above, was slowly added to stirred saturated aqueous Na salicylate (750 ml.). Foaming was reduced to a minimum by stirring slowly and adding a few drops of capryl alcohol. The mixture was stirred for 30 min. at room temperature and a slightly turbid, dark-red solution, pH 6·5–7·5, was formed. On spinning the solution at 2000 rev./min. for 15 min. in an angle centrifuge, a deposit, which was obviously foreign matter, separated. The yield of this material, after washing free from salicylate with ethanol and freeze drying, was 0·1–0·2 g. In the biological assay different samples did not behave consistently. Some had no action, others were very toxic, and still others showed some virulence enhancement, but this was never greater than that of the original crude active product. To make certain all foreign matter was removed, the centrifugate was filtered through a battery of six no. 54 Whatman filter papers (11 cm.) into flasks immersed in an ice and salt freezing mixture. Filtration took about 30 min. by which time the filtrate had cooled to 0°. Absolute industrial ethanol (5 vol.) at −18° was added and the mixture kept at −18° overnight. The precipitate was collected and washed with successive quantities (400 ml.) of cold 90% industrial ethanol until free from salicylate ($FeCl_3$) and freeze dried. Yield 7·5–9·0 g. Larger quantities of crude active material were dealt with in 15 g. batches up to the precipitation with ethanol; batches were then combined for washing free from

salicylate, using the Sharples centrifuge. Table 2 shows the activities of a few different batches prepared in this manner. These results, and those of the fractionations below, indicate the absence of any large-scale destruction of activity in saturated aqueous Na salicylate under these conditions.

Table 2. *Activities of some fractionation samples before and after filtration in saturated aqueous sodium salicylate and precipitation with ethanol*

Exp. no.	Activity of initial crude mucin ('Wilson units')	Yield of filtered material (%)	Activity of filtered material ('Wilson units')
1	2·53 (2·09–3·08)	58	2·77 (2·20–3·50)
2	1·71 (1·14–2·55)	60	1·55 (0·77–2·88)
3	1·71 (1·14–2·55)	58	1·60 (1·03–3·46)

Properties of the salicylate filtered products. These were grey powders almost insoluble in water at pH 7; the aqueous suspensions were non-viscous, and contained the suspended material in a very fine state of subdivision. Two different samples had respectively, N (Dumas), 14·5 and 13·5, and sulphated ash, 5·4 and 6·9%, calculated with reference to the substance dried over P_2O_5 at 0·1 mm. Hg at 60°. The outside chance of chemical combination between active material and Na salicylate during the removal of foreign matter was ruled out by the following experiments. Three different samples (0·1 g.) were hydrolysed with 5 ml. of N-HCl for 1 hr. at 100°, followed by cooling and neutralizing to phenol red. The same procedure was carried out using 5 ml. of N-NaOH on 0·1 g. samples. After standing overnight, the clear supernatant fluids gave no colour with $FeCl_3$ solution. A deep violet colour was obtained when Na salicylate (2 mg.) was included in identical tests.

Attempted fractionations of the active product in aqueous sodium salicylate solutions

Exhaustive attempts were made to fractionate the active material from aqueous Na salicylate. The fractionations effected a degree of separation, but this was not sufficient to warrant their inclusion in any general process of purification.

Fractionation of the crude active material with different strengths of aqueous sodium salicylate. High-speed centrifugation of saturated salicylate solutions deposited a small amount of slime; the amount of slime could be increased by lowering the salicylate concentration. 5 g. batches of crude active product (activity, 1·31 (1·14–1·57) 'Wilson units') were dissolved in 250 ml. each of saturated, 90% saturated and 80% saturated Na salicylate solutions, and spun at 2000 rev./min. The supernatants were not filtered, but spun for 1 hr. at 26,000 rev./min. in the closed bowl of the Sharples. The clear fluid was cooled to 0° and mixed with absolute industrial ethanol (10 vol.) at −18°. After remaining overnight at −18° the precipitate was collected, washed free from salicylate and freeze dried. The slime left in the centrifuge bowl was cooled to 0° and mixed with absolute industrial ethanol (500 ml.) at −18°. The precipitate was collected and dried as above. Table 3 shows the results of these experiments. A tendency for insoluble fractions to have a greater activity than either the original sample or the soluble fractions was indicated.

Fractionation with ethanol of saturated aqueous sodium salicylate solutions of the filtered active material. Filtered active material (2·5 g., activity, 1·55 (0·84–2·61) 'Wilson units') was dissolved in saturated aqueous Na salicylate (50 ml.). The solution was cooled to 0° and ethanol (1 vol.) at −18° slowly added. After standing for 2 hr. at 0°, the bulky precipitate was spun off at 0° and washed twice with 1 part saturated aqueous Na salicylate and 1 part ethanol (25 ml.). When washed free from Na salicylate and freeze dried the yield was 1·5 g. Activity, 1·91 (1·35–2·68) 'Wilson units'. A further volume of ethanol was added under the same conditions and the precipitate (0·35 g.) had an activity of 2·43 (1·54–5·43) 'Wilson units'. Final precipitation was made using 10 vol. ethanol; this material (0·3 g.) had activity 0·99 (0·32–1·99) 'Wilson unit'.

Table 3. *Yields and activities of products fractionated in different strengths of aqueous sodium salicylate*

(Initial crude sample 1·31 'Wilson units' (1·14–1·57).)

Sodium salicylate solution (% saturation)	Insoluble material: Yield (%)	Insoluble material: Activity ('Wilson units')	Soluble material: Yield (%)	Soluble material: Activity ('Wilson units')
100	12	2·29 (1·43–2·58)	54	1·84 (1·33–2·87)
90	18	2·47 (1·71–3·59)	46	1·34 (0·96–1·77)
80	23	2·10 (1·34–2·99)	42	1·35 (0·90–1·92)

The marked increase of activity of purified fractions when suspended in inactive solutions of semi-pure blood-group substance

If active fractions obtained by the above procedure were suspended in an inactive solution of semi-pure blood-group substance, a three- to four-fold increase in activity resulted. The semi-pure blood-group substance was the material soluble at pH 4·2 which was precipitated with ethanol in earlier work. Full details of these results, which involve different combinations of two different batches of inert semi-pure blood-group substance with two purified virulence-enhancing fractions are given in Table 4.

The same marked increase of the activity of purified fractions when suspended in solutions of tragacanth and agar to give approximately the same viscosity as with semi-pure blood-group substance

The high viscosity of suspensions with semi-pure blood-group substance, as compared with aqueous suspensions, suggested the use of other viscous

Table 4. *Comparison of the activities of two virulence-enhancing fractions when suspended (a) in water and (b) in inert semi-pure blood-group substance solutions*

(The results were obtained using the normal biological assay procedure (see previous paper) combining the injection solutions with a constant number of *Bact. typhosum*. Controls without organisms were carried out on batches of twenty mice for all injection solutions; not more than one mouse died in any of these controls. Normal results were obtained for the 'standard' mucin death rates, organisms control death rates and bacterial counts; these results are not included in the table. Two virulence-enhancing fractions Mucin 100 and Mucin 131 were used and two different samples of semi-pure blood-group substance, abbreviated Bgs. *A* and Bgs. *B*.)

Solution injected with 5000 *Bact. typhosum*: Nature and strength (w/v) of the virulence-enhancing fraction used	Suspending medium	Death rates in batches of 20 mice in one or more experiments (%)	Activities of virulence-enhancing fractions 'Wilson units' obtained by direct comparison with the 'standard' preparation
Mucin 100 0·75%	Water	15, 25, 30	1·53 (1·28–1·83)
Mucin 100 0·25%	Water	Nil	
Mucin 100 0·75%	Bgs. *A*, 3·5% (w/v)	80, 95	5·91 (5·06–6·90)
Mucin 100 0·25%	Bgs. *A*, 3·5% (w/v)	40, 45, 20	
Mucin 100 0·25%	Bgs. *B*, 3·5% (w/v)	35	5·11 (4·04–6·43)
Mucin 131 0·6%	Water	45, 60	2·77 (2·20–3·50)
Mucin 131 0·2%	Water	Nil	
Mucin 131 0·2%	Bgs. *A*, 3·5% (w/v)	40, 45	7·99 (5·60–11·97)
Nil	Bgs. *A*, 3·5% (w/v)	Nil	—
Nil	Bgs. *B*, 3·5% (w/v)	5	—

Table 5. *Comparison of the activities of two virulence-enhancing fractions when in suspensions of approximately the same viscosity using semi-pure blood-group substance, tragacanth and agar*

(Experimental arrangements and materials: see Table 4.)

Solution injected with 5000 *Bact. typhosum*: Nature and strength (w/v) of the virulence-enhancing fraction used	Suspending medium	Experiments on direct comparison of the same concentration of active sample in different suspensions: Death rates in batches of 20 mice (%)	Viscosities at 37° relative to the Bgs. *A*, 3·5% (w/v) suspension	Separate experiments, each suspension compared directly with the 'standard' mucin preparation: Activities ('Wilson units')	Viscosities at 37° relative to the Bgs. *A*, 3·5% (w/v) suspension
Mucin 100 0·2%	Water	Nil	—	—	—
Mucin 100 0·2%	Bgs. *A*, 3·5% (w/v)	60	1·0	5·91 (5·06–6·90)	1
Mucin 100 0·2%	Tragacanth, 0·17% (w/v)	40	0·9	6·64 (5·12–8·44)	1·1
Mucin 100 0·75%	Water	20	—	1·53 (1·28–1·83)	—
Mucin 100 0·75%	Bgs. *A*, 3·5% (w/v)	95	1·0	—	—
Mucin 100 0·75%	Tragacanth, 0·17% (w/v)	95	0·6		
Mucin 131 0·6%	Water	48	—	2·77 (2·20–3·50)	—
Mucin 131 0·2%	Water	Nil	—		
Mucin 131 0·2%	Bgs. *A*, 3·5% (w/v)	45	1·0	7·99 (5·60–11·97)	1
Mucin 131 0·2%	Tragacanth, 0·17% (w/v)	75	1·1	8·46 (5·97–11·05)	0·8
Mucin 131 0·2%	Agar, 0·15% (w/v)	60	0·7	8·28 (6·94–9·87)	0·8
Nil	Bgs. *A*, 3·5% (w/v)	Nil	—	—	—
Nil	Tragacanth, 0·17% (w/v)	Nil	—	—	—
Nil	Agar, 0·15% (w/v)	Nil	—	—	—

solutions as media for carrying the virulence-enhancing fractions. The same marked increase in virulence-enhancing activity was observed with tragacanth, and in smaller scale experiments, with agar.

Preliminary experiments were done to ascertain the concentrations of tragacanth and agar necessary to give suspensions of the active fractions with approximately the same viscosity as the semi-pure blood-group substance suspension. These viscosities were determined at 37° in an Ostwald viscometer, after the suspensions had been heated at 60° for 0·75 hr. (killing vegetative organisms), and just before adding the bacterial suspensions. Full details of the results of this work, which involved two different virulence-enhancing samples, are given in Table 5.

DISCUSSION

The first part of the work described in this paper is conclusive evidence that blood-group substances *per se* are not responsible for the virulence-enhancing activity of hog gastric mucin. This was indicated by the lack of parallelism between the virulence-enhancing activity and the blood-group activity of samples from individual hog stomach linings; it was proved by the separation of an active virulence-enhancing fraction from the blood-group mucoid, by dialysis at pH 4·2. The separation was confirmed by fractionation of the mucin extracted with 0·1 N-Na_2CO_3 solution.

After autolysing the minced mucosae at pH 3 some material remains insoluble and is discarded in the preparation of crude blood-group substance. Large yields of a product having double the activity of the original blood-group substance were obtained by a method of preparation which included this insoluble matter in the dialysis precipitate at pH 4·2, while removing the fat present in it. Extraction of this material at pH 6 removed much extraneous material and a more active product was obtained. These results were embodied in a method of preparing a bulk product of relatively high activity, direct from hog stomach linings.

Up to the present, the active fractions have always been obtained as insoluble solids, and consequently were contaminated with foreign matter. To remove this foreign matter, and to assist in further fractionation, many solvents were investigated. Urea (40 %), phenol (90 %) and cresol were examined in some detail, but always a residue remained which constituted over 20 % of the initial material. This residue always proved to be active: it is impossible as yet to say whether this effect is due to the sparing solubility of the active principle, or to its strong adsorption on the residue. Eventually success was achieved using saturated aqueous sodium salicylate, followed by precipitation with ethanol.

The active products thus obtained were practically insoluble in water at pH 7·0–7·2, forming non-viscous suspensions. They were too heterogenous for detailed chemical analysis, but the nitrogen (Dumas) figure suggests a protein or peptide nature.

The most active fractions which could be obtained consistently by the above procedures had most probable activities of 2·5–3·0 'Wilson units'. This was a concentration of seven to nine times the activity of the original crude blood-group substances. In view of the methods described and the yields obtained, this concentration of activity was low. Therefore, the possibility that other factors present in the crude mucin were necessary for a full manifestation of the virulence-enhancing activity, was considered. The material chosen for experiment was the soluble fraction which had been separated from the active substance insoluble at pH 4·2; it had been precipitated with ethanol, and contained blood-group activity, but had negligible virulence-enhancing power. This substance was designated semi-pure blood-group substance. In 3·5 % (w/v) solution it had no inherent virulence-enhancing activity, but its viscosity was of the same order as that of the active solutions of crude blood-group substances. The virulence-enhancing activity of salicylate-filtered fractions, in aqueous suspension, was increased three- to fourfold when suspended in the above solution. This was confirmed using different batches of virulence-enhancing fraction, and of semi-pure blood-group substance. The concentration of activity was therefore the more reasonable figure of 25–30 times.

Hence it was shown that the virulence-enhancing power of hog gastric mucin was not due to one factor alone, as reported or tacitly assumed in previous work, but to a combination of two factors. These were the more important virulence-enhancing factor itself, and also an inert factor or factors present in the crude and semi-pure blood-group substance, which were necessary for the full manifestation of the activity of the virulence-enhancing factor.

Attention was now turned to the nature of this adjuvant action. The most obvious difference between the aqueous suspensions and the semi-pure blood-group substance suspensions was the marked viscosity of the latter (10–15 times greater). In order to discover whether the adjuvant effect was connected with the viscosity, suspensions of the virulence-enhancing factor were made using solutions which would give approximately the same viscosity at 37° as the semi-pure blood-group suspension. Tragacanth, and agar in smaller scale experiments, were used at very low concentrations (0·17 % (w/v) and 0·15 % (w/v), respectively) to give the required viscosity; these solutions showed no virulence-enhancing activity alone. Again, a marked increase in activity occurred when these solutions were used to suspend the virulence-enhancing factor, and, within the limits of the biological assay, this increased activity was the same as that obtained with the suspensions in semi-pure blood-group substance.

These experiments suggest that the materials causing the viscosity of hog gastric mucin are those necessary for the full manifestation of the activity of the virulence-enhancing factor. The presence of blood-group substances is undoubtedly the cause of some, if not all, of the viscosity. The reported virulence-enhancing activity of some samples of blood-group substances may well have been due to the presence of a small amount of virulence-enhancing factor exerting its maximum effect in the viscous medium.

The viscosity of the medium probably delays the absorption and inactivation of the active factor by

the host at the site of injection. If this is so, not only is the initial viscosity important, but also the degree to which it is maintained under the influence of the body fluids of the host. With reference to this delay in absorption, it is relevant to point out that Olitzki & Koch (1945) have reported that adsorbents such as kaolin increase the action of mucins in enhancing virulence. Also, it is interesting that extremely viscous media such as 2, 4 and 10 % (w/v) agar (Anderson & Oag, 1939), 6 % (w/v) starch paste (Robertson & Fox, 1939), 2·5 % (w/v) tragacanth (Steinberg, 1931) and 5 % (w/v) tragacanth (Benians, 1924), have virulence-enhancing effects alone which are, however, far smaller than that of mucin.

Future work on the virulence-enhancing factor of mucin, therefore, falls into two parts: the more important fractionation of the virulence-enhancing factor itself, and the investigation of the other factors necessary for the full manifestation of its activity. The work is being continued along these lines.

SUMMARY

1. Preliminary fractionations have been carried out on preparations from fresh hog stomach linings for a factor responsible for the virulence-enhancing power for bacteria.

2. Crude blood-group substance prepared by autolysis had virulence-enhancing activity which, in the same samples, did not run parallel with the blood-group activity.

3. Dialysis at 0° at pH 4·2 separated a small amount of active insoluble material from inactive soluble blood-group mucoid; the latter was precipitated in a semi-pure state by ethanol. More of this active material was prepared directly from fresh linings and further purified.

4. The still heterogeneous active material contained nitrogen (Dumas) 14·5, 13·5%; in aqueous suspensions, which were non-viscous, different batches consistently had activities seven to nine times those of the crude blood-group substance.

5. This activity, however, was increased three- to fourfold when suspended in a completely inactive 3·5 % (w/v) solution of semi-pure blood-group substance (see above), which imparts a marked viscosity to the suspension. This viscosity was of the same order as that of the original active crude blood-group substance solution; a concentration of activity of 25–30 times is therefore implied.

6. The same marked increase in activity was produced by suspending the active material in tragacanth and agar solutions to give suspensions with approximately the same viscosity at 37° as the above.

7. The virulence-enhancing power of hog gastric mucin is due to a virulence-enhancing factor which is active alone. But it only manifests its full activity when in combination with a second inert factor or factors, which may well act by providing a viscous medium for its suspension.

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On the State of Hyaluronic Acid in Synovial Fluid

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Synovial fluid contains, besides proteins and salts, a polysaccharide of the type termed 'hyaluronic acid' (Meyer, Smyth & Dawson, 1939). This contains acetylglycosamine and glucuronic acid, and it has been claimed that these substances, in equimolar proportions, compose the whole of hyaluronic acid. In the past the isolation of hyaluronic acid from synovial fluid and from other sources has involved the use of relatively vigorous methods; our aim has been, by use of the mildest possible procedure, to find in what state hyaluronic acid occurs in native synovial fluid, that is, whether it is a separate component or is combined with other material.

It was found possible to separate the whole of the hyaluronic acid from synovial fluid by ultrafiltration, in the form of a complex which sedimented as a single component in the ultracentrifuge, giving a very sharp sedimentation boundary. It contained a constant amount of protein and its glucosamine content was possibly rather less than would correspond with an equimolar ratio of acetylglucosamine and glucuronic acid.

The high specific viscosity of solutions containing hyaluronic acid and the sharpness and rate of sedimentation of the boundary in the ultracentrifuge are properties likely to be affected by any degradative change which occurs during isolation. Synovial fluid, mucin, the material prepared by ultrafiltration and the material prepared by precipitation with ethanol have therefore been compared with respect to these properties. Samples of the ultrafilter residue, of mucin and of ethanol-precipitated material were obtained which contained undegraded hyaluronic acid, as judged by these criteria, though the formation of mucin tended to cause degradation. In every sample which was undegraded, the composition of the hyaluronic acid complex was the same, and any further reduction of the proportion of protein was always accompanied by signs of degradation. It is concluded, therefore, that hyaluronic acid occurs in synovial fluid as a definite complex with protein. The physico-chemical methods used have enabled certain physical characteristics of this complex to be measured.

METHODS

Collection and storage of synovial fluid. The fluid was withdrawn into a syringe through a lumbar puncture needle from the knee and ankle joints of the hindlimbs of cattle, 15–30 min. after slaughtering. The fluid from the ankle was withdrawn, after the skin had been removed, by introducing the needle into the sac-like dilatation of the joint cavity immediately anterior to the Achilles tendon. The knee-joint fluid was drawn from the supra-patellar pouch after the joint had been opened. Average volumes obtained were 20 ml. from the ankle joint and 5 ml. from the knee joint. Any fluid which was contaminated with a large amount of blood was discarded. The fluid was centrifuged to remove any blood or connective tissue, and then filtered through muslin. Preliminary experiments showed that fluid could be kept for several months at 1°, with short periods of exposure to room temperature, without any change of viscosity. At room temperature the viscosity fell markedly within 24 hr., owing to infection with bacteria; the addition of toluene considerably improved its stability at room temperature. All extended experiments were therefore done in the cold room and no special precautions were taken to ensure sterility.

Glucosamine. A modification of the method of Elson & Morgan (1933) was used, using the quantities of reagents and the hydrolysis procedure described by Palmer, Smyth & Meyer (1937). The procedure after hydrolysis differed somewhat from that of Palmer *et al.* (1937), because of the small quantities of glucosamine (0·1–0·5 mg.) which it was required to estimate. In order to avoid diluting the hydrolysate more than necessary, it was made up in a standard flask to 10 ml., of which 4 ml. were taken for the preliminary titration with NaOH. A further 4 ml. were then neutralized and made up to 10 ml., of which 1 ml. was taken for the glucosamine estimation. The sensitivity of the method was further increased slightly by increasing the acetylation time from 15 to 30 min.; a preliminary experiment had shown that the maximum amount of glucosamine was acetylated after 30–45 min. and that longer times led to loss (Fig. 1). It was found that a variable amount of acetylacetone was lost by evaporation during the acetylation, and the technique of Palmer *et al.* (1937), of exposing the necks of the

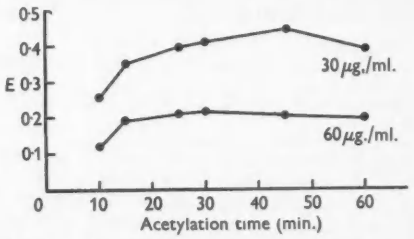

Fig. 1. Variation of glucosamine colour with acetylation time. Extinction coefficients E measured at 535 mμ.

flasks to a strong current of air, did not prevent this. Since this loss would be more serious with a longer acetylation time, a water condenser was made through which the necks of the flasks protruded, while their bulbs were immersed in a boiling water bath. This reduced evaporation to a small and constant amount, and markedly improved the reproducibility of the results. In most of the determinations, the method of Palmer *et al.* (1937) was followed, the standard glucosamine solutions being introduced after the hydrolysis stage. Later, these standards were compared with standards subjected to the hydrolysis procedure; the recovery after hydrolysis was rather variable, being from 72 to 96%, and averaging 85%. After this was discovered, hydrolysed standards were always used. The earlier values were corrected

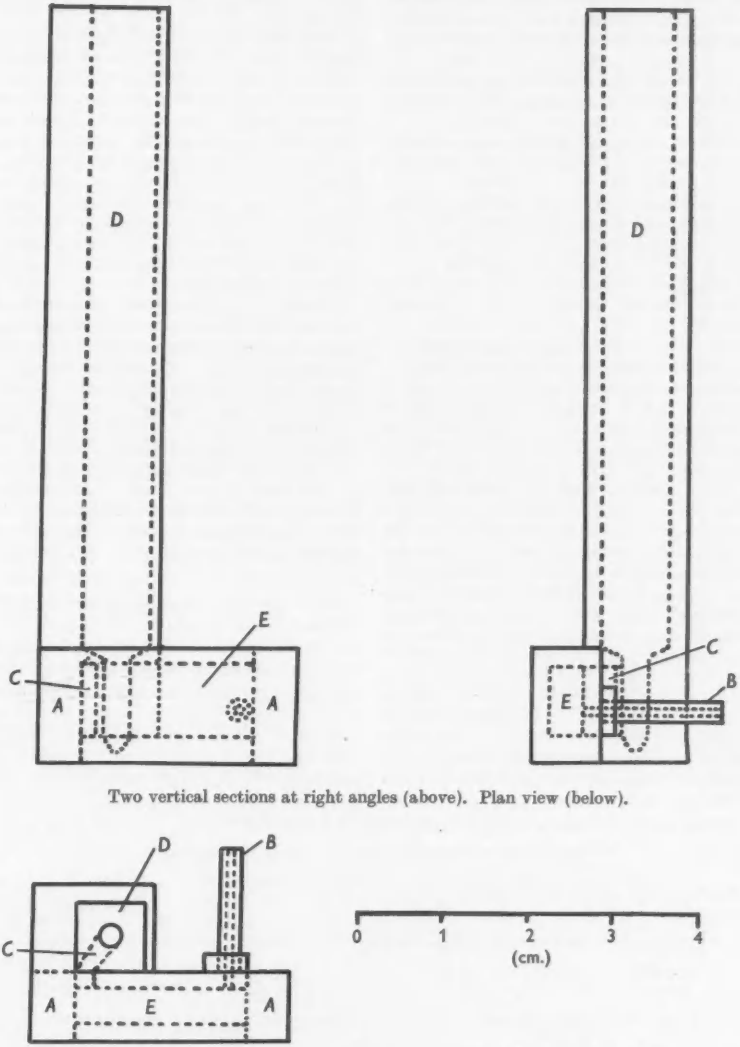

Fig. 2. Diagram of end-flow diffusion cell (the water jacket is not shown). The cell is of Perspex, except for the glass optical faces *A*, the glass outflow tube *B* and the pipette (not shown) which fits into the column *D*. The solution and buffer flow from the bottom of *D* through the channel *C* into the tunnel *E* and out through *B*, the boundary being established in *D*, *C* and *E* at the level of *B*.

on the assumption that the recovery of glucosamine from the unknown solutions also averaged 85%. Extinctions were measured at 535 mμ. in a Beckman spectrophotometer.

Total nitrogen. Total N was estimated by micro-Kjeldahl, using $CuSO_4$ and K_2SO_4 as catalyst, and 5–6 hr. digestion. Use of selenium catalyst and of 8 hr. digestion gave the same results.

Dry weight. Dry weights were determined by heating the material in a weighed crucible in an electric oven at 105° to constant weight.

Refractive increment. A simple double prism refractometer (designed by Dr J. St L. Philpot) was used, with light of 546 mμ.; its accuracy was 10^{-6} of refractive index, corresponding to a concentration of about 0·0005 g./100 ml. The specific refractive increments of materials were determined and the refractive increments of dialysed solutions were subsequently used to estimate concentrations.

Ultraviolet absorption. Ultraviolet absorption was measured between 240 and 400 mμ. using a Beckman spectrophotometer.

Ultracentrifuge. A Svedberg oil-turbine ultracentrifuge with a Philpot (1938) optical system was used. The procedure of running and the methods of calculating the sedimentation constants and of estimating the quantities of components were as described by Cecil & Ogston (1948). All materials were dialysed against a standard buffer: NaCl, 0·2M; Na_2HPO_4, 0·0077M; KH_2PO_4, 0·0023M; pH 7·3.

Diffusion. Diffusion was measured with a Gouy diffusiometer (Coulson, Cox, Ogston & Philpot, 1948), with a modified cell (Fig. 2). In this cell the flow of solution and solvent takes place along, instead of across, the optic axis, from a concentric reservoir at one end to a circular outflow tube at the other. The level of the boundary adjusts itself so that the cross-section of the outflow tube is available to solution and solvent in proportion to their viscosities; adjustment of the boundary stop of the optical system must be made when it has been seen at what level the boundary is formed by a given solution. This cell gave correct values for the diffusion constants of glycine and of lactoglobulin. In spite of the adaptation of the cell for viscous solutions, it was found that, in order to form a satisfactory boundary, the concentration of hyaluronic acid must not exceed 5 mg./ml. and a considerable amount of free protein must also be present. For example, it was not possible to obtain a boundary with hyaluronic acid prepared by ethanol precipitation. Analysis of the records was made by the method of Ogston (1949).

Viscometry. Routine measurements of viscosity were made with an Ostwald viscometer with flow time for water of about 12 sec.; 2 ml. of fluid was required. All measurements were made at 25°. In order to study the anomalous viscosity of solutions containing hyaluronic acid, a Couette viscometer was designed and made in this laboratory, modified from that described by Lawrence, Needham & Shen (1944). The fluid (10 ml.) was contained in the annular gap (0·148 cm.) between concentric cylinders. The outer cylinder was rotated at speeds up to 150 rev./min., giving rates of shear numerically 0·5 times the speed in rev./min. The inner cylinder was suspended by a torsion wire and its deflexion was measured.

Estimation of the concentration of various materials. It was convenient to relate the properties of synovial fluid and its derivatives to their concentrations of hyaluronic acid glucosamine per ml. The glucosamine contents of ultrafilter residues and of ethanol-precipitated hyaluronic acid were measured directly. Since synovial fluid contains considerable amounts of non-hyaluronic acid glucosamine, it was necessary to estimate glucosamine in mucin derived from it. The mucin glucosamine represents all the hyaluronic acid glucosamine of synovial fluid, and was found to be constant: the weight of mucin, determined directly or refractometrically, could therefore be used to estimate hyaluronic acid glucosamine in synovial fluid.

EXPERIMENTS AND RESULTS

Chemical composition of synovial fluid. Preparation and chemical composition of its derivatives

(1) *Synovial fluid*

The viscosity of each sample of synovial fluid was measured in the Ostwald viscometer immediately after collection and the maintenance of this viscosity

Table 1. *Analysis of synovial fluid*

(Numbers of samples analysed are given in brackets.)

(*a*) Total N, mucin N, and wt. of mucin (mg./100 ml.) of synovial fluid

	Total N		Mucin N		Mucin		Mucin N (% of mucin)	
Source	Range	Mean	Range	Mean	Range	Mean	Range	Mean
Ankle	128–360	182 (10)	16–39	24 (6)	142–455	244 (11)	9·9–12·3	11·1 (6)
Knee	249–373	311 (11)	34–47	40 (8)	320–510	408 (14)	9·8–11·6	10·8 (8)

(*b*) Total fluid glucosamine in relation to mucin (mg./100 ml.) of synovial fluid

	Mucin		Total glucosamine		Total glucosamine (% of mucin)	
Source	Range	Mean	Range	Mean	Range	Mean
Ankle	180–205	193 (2)	36–42	39 (2)	20·2–20·6	20·4 (2)
Knee	405–446	426 (2)	86–87	86·5 (2)	19·3–21·5	20·4 (2)

(*c*) Mucin glucosamine in relation to mucin

No. of precipitations of mucin ...	1	1	·2	4
Mucin glucosamine (% of mucin) ...	7·3	8·4	8·9	10·0

Mean specific refractive increment of mucin = 0·00164

was used as a criterion of its stability on storage, any sample which showed a fall of viscosity being rejected. The spontaneous precipitation of mucin during storage, described by Davies (1944), was never observed. The fluid was yellow and highly viscous, that from the knee joint being smaller in volume, of darker colour and more viscous than that from the ankle joint: the knee-joint fluid contained a higher percentage of hyaluronic acid and of protein. Some analyses of fluid are given in Table 1. The mucin clot contains all the hyaluronic acid of the fluid. Since, however, mucin contains about 8% of glucosamine, while the table shows that the total glucosamine of the fluid may be 20% (w/w) of the mucin, only about half the total glucosamine of synovial fluid is accounted for by hyaluronic acid. This was confirmed by precipitating and washing mucin from a sample of fluid; the mucin was found to contain 49% of the total glucosamine and precipitation of the residual solution with trichloroacetic acid removed a further 46% of the total glucosamine, leaving only 5% in solution. It appears, therefore, that most of the non-hyaluronic acid glucosamine is associated with protein, which would contain about 1·5% of glucosamine.

Synovial fluid, dialysed against the standard buffer (Fig. 7*a*), gave a sedimentation diagram identical with that of undialysed fluid. Three boundaries were present: a very sharp boundary due to the hyaluronic acid complex, whose rate of sedimentation varied rapidly with concentration, and two boundaries whose rates of sedimentation (S_{20} about 4 and 6×10^{-13}) were typical of the main albumin and globulin components of normal serum. In one case the relative proportions of these latter components were measured. The combined concentration was found to be 1·13 g./100 ml. and the albumin/globulin (A/G) ratio 4·4. Bauer, Ropes & Waine (1940) found by salting-out the A/G ratio of synovial fluid to be 3·9.

(2) *Mucin*

Preparation. A compact clot of mucin is formed when synovial fluid is acidified with acetic acid to pH 4–5. On a small scale, 1 ml. of synovial fluid was washed from an Ostwald pipette into 1 ml. of distilled water and about 0·06 ml. of 10% (w/v) acetic acid was added. The entire clot was collected on a glass rod and washed six times with 1% (w/v) acetic acid, and then twice with distilled water. It was carefully scraped off into a weighed crucible and dried at 105° to constant weight. The dried residue could then be used for determining total N. For preparation of mucin on a larger scale, the method of Ropes, Robertson, Rossmeisl, Peabody & Bauer (1947) was followed. After a suitable number of washings and reprecipitations, the mucin was dissolved in $NaHCO_3$ or Na_2HPO_4 and dialysed against the standard buffer.

Composition. Mucin collected and analysed without washing contained entrained protein up to 60–90% of the final weight of washed mucin; successive washings on the small scale removed this protein, and after six washings no further diminution in the weight of the clot occurred. Some analyses of mucin are given in Table 1. It can be seen that the glucosamine content of washed mucin tended to be higher if the mucin had been several times reprecipitated.

The sedimentation diagram (Fig. 7*b*) resembled that of synovial fluid except that relatively less protein was present and this consisted of a single component which sedimented at the rate of serum albumin. The amount of this component was estimated from the ultracentrifuge diagrams and was compared with the total refractive increment of the solution. The refractive increment due to hyaluronic acid was calculated from the glucosamine content and the specific refractive increment found from experiments on the ultrafilter residue (see below).

The combined refractive increments of the free albumin and of the hyaluronic acid were found to fall short of the total refractive increment (Table 2) by an amount considerably greater than could be accounted for by experimental errors. It was concluded, in view of later experiments, that this difference can be accounted for by protein which forms a complex with the hyaluronic acid. The total nitrogen also was in excess of that accounted for by free albumin plus glucosamine, and this excess corresponded with the amount of combined protein deduced from refractive measurements. The proportions of hyal-

Table 2. *Components of mucin*

Mucin sample	12*K*	18*A*	21
(*a*) Glucosamine (mg./ml.)	0·22	0·47	0·35
(*b*) Refractive increment of hyaluronic acid (=glucosamine × 100/32 × 0·000144)	0·000100	0·000213	0·000159
(*c*) Refractive increment of free protein	0·000279	0·000543	0·000426
(*d*) Refractive increment of hyaluronic acid + free protein	0·000379	0·000756	0·000585
(*e*) Total refractive increment	0·000420	0·000836	0·000670
(*f*) Refractive increment of protein in complex (=*e* − *d*)	0·000041	0·000080	0·000085
(*g*) Fraction of free protein (=*c*/(*c* + *f*))	0·87	0·87	0·83
(*h*) Proportion of complex in total (=(*b* + *f*)/*e*)	0·34	0·35	0·36
(*i*) Proportion of hyaluronic acid in complex (=*b*/(*b* + *f*))	0·71	0·73	0·65
(*j*) Proportion of protein in complex (=*f*/(*b* + *f*))	0·29	0·27	0·35

uronic acid and protein which form this complex have been calculated and are given in terms of refractive increment in the last two rows of Table 2.

Selection of protein in the formation of mucin. Separated hyaluronic acid (Meyer & Palmer, 1936) will form mucin clots with a variety of proteins. It was confirmed that the ultrafilter residue will do so with serum albumin, serum globulin, haemoglobin and lactoglobulin. On the other hand, the mucin prepared from synovial fluid contains albumin exclusively. An experiment was performed in which lactoglobulin was added to a sample of synovial fluid in sufficient amount to form a mucin clot with the whole of the hyaluronic acid present. Mucin formed from this mixture was shown in the ultracentrifuge to contain only serum albumin.

(3) *The ultrafilter residue*

Preparation. Gradocol graded collodion membranes, obtained from St Mary's Hospital, were used for a few preparations. All the hyaluronic acid was retained by membranes of average pore diameter (A.P.D.) 0·19 μ., and most of it by membranes of A.P.D. 0·52 μ. The free protein passed through readily. In later preparations Jena '5 on 3' sintered glass filters, of A.P.D. about 0·6 μ. were used, and almost completely retained the hyaluronic acid. These proved to be much faster than the collodion membranes and showed less tendency to become clogged. Their only disadvantage was that, when the residue on the filter was resuspended, a certain amount of glass powder was always scraped off, even by a rubber-tipped rod.

About 20 ml. of synovial fluid or redissolved mucin was placed in each glass filter; the filter was covered with a lid and connected to an evacuated reservoir. Filtration was complete after about 24 hr. The residue was then scraped off the filter and resuspended *in situ* in distilled water; when washing with saline, an equal volume of 0·4 M-NaCl was then added. Usually six washings with 0·2 M-NaCl were given and then twenty or more with distilled water. The whole process was carried out in the cold room. The removal of protein was followed by examining small samples of resuspended material. Their concentrations were determined refractometrically, and the amount of protein was estimated by ultraviolet absorption in 0·1 N-NaOH; the difference of extinctions at 285 and 330 mμ. (the latter to allow for haze: Holiday & Ogston, 1938) was taken as a measure of the protein. Correlation between total N and ultraviolet absorption of the final products was satisfactory (Table 3).

In no case was it found possible to remove all the protein by this method. In two cases (16*K* and 23) washing was continued until no further fall in the ultraviolet absorption could be produced; in other cases (19*K*(1) and 22*K*) washing was stopped short of this point.

The material finally obtained was quite colourless and most of it went into solution. The undissolved solid, including suspended glass, could be removed by centrifuging at 4500 *g*. The removal of suspended solid was found not to have changed the composition significantly with respect to total N and glucosamine; subsequently all analyses were made on the cleared solution.

Composition. Exhaustively washed samples showed a single boundary in the ultracentrifuge (Fig. 7*d*). Nitrogen and glucosamine values for a number of ultrafilter residues are given in Table 3, together with a summary of their physico-chemical properties. 19*K*(1), 22*K* and 23 were samples in which both the viscosity and sedimentation boundary were normal, and can therefore be regarded as containing undegraded hyaluronic acid. Of these, 22*K* contained some free protein which sedimented separately in the ultracentrifuge, and this is confirmed by the high values for nitrogen and ultraviolet absorption.

By partition chromatography (see below) it appeared that the non-hyaluronic acid component of the ultrafilter residue was a protein of normal composition. This was therefore assumed to have a normal nitrogen content of 15·5%, and the non-glucosamine nitrogen values were multiplied by 6·45 to give values for protein. The amount of hyaluronic acid was taken to be the total weight less protein,

Table 3. *Composition of ultrafilter residues and of ethanol-precipitated hyaluronic acid*

	Ultrafilter residues						Precipitated hyaluronic acid	
Sample no.	16*K*	19*K*(*c*)	19*K*(1)	19*K*(2)	22*K*	23	23	23
Filter	Glass	Collodion	Glass	Glass	Glass	Glass	—	—
No. of washings	38	27	19	39	22	25	—	—
No. of precipitations	—	—	—	—	—	—	3	5
Total N (%)	5·4	3·7	6·9	5·4	10·0	7·0	7·0	4·8
$E_{285}-E_{330}$ at 10 mg./ml.	0·265	0·32	0·54	0·39	0·79	0·44	0·54	0·20
Glucosamine (%)	26	25	24	20	14	18	32	32·5
Non-glucosamine N (%)	3·4	1·8	4·9	3·8	8·9	5·6	4·5	2·1
Protein (%, non-glucosamine N × 6·45)	22	11	32	24	57	36	29	14
Non-protein (%)	78	89	68	76	43	64	71	86
Glucosamine (% of non-protein fraction)	33	29	36	27	33	27	45	38
Total specific refractive increment × 10^5	151	151	152	156	167	156	155	146
Specific refractive increment of non-protein fraction	143	147	139	148	149	143	145	140
Sedimentation constant	—	Normal	Normal	Normal	Normal	Normal	Normal	—
Non-protein sedimentation boundary	—	Diffuse	Sharp	Diffuse	Sharp	Sharp	Sharp	—
Viscosity	Normal	Low	Normal	Low	Normal	Normal	Normal	Low

and the glucosamine was finally expressed as a percentage of hyaluronic acid.

It can be seen that the glucosamine constituted 27–36% of the hyaluronic acid. In the two normal samples known to contain no free protein, 19*K*(1) and 23, the combined protein constituted 32 and 36% of the ultrafilter residue. Degradation in samples 19*K*(*c*) and 19*K*(2), shown by the low viscosity and diffuse sedimentation boundary, was associated with an abnormally low percentage of non-hyaluronic acid nitrogen. 16*K*, however, was physico-chemically normal, although its non-hyaluronic acid nitrogen was low. In 22*K* the free protein was found to have the sedimentation constant of serum globulin. This was to be expected, since albumin passes through a fine filter more rapidly (Fig. 7*c*). Measurement of this free protein boundary in the ultracentrifuge diagram and calculation of its contribution to the total nitrogen showed that the nitrogen and glucosamine contents of the hyaluronic acid complex were 6·8 and 22% respectively.

A paper chromatogram of hydrolysate of 16*K* was made for us by Mr D. S. Parsons. This showed the presence of the following amino-acids: aspartic and glutamic acids, glycine, alanine, tyrosine, tryptophan and phenyl alanine, and possibly traces of serine and proline. The presence of tyrosine and tryptophan was also indicated by the ultraviolet absorption of the ultrafilter residue.

Mucin formation by the ultrafilter residue. Ultrafilter residues containing 7% of nitrogen gave only slight cloudiness when the solution was acidified. After addition to the ultrafilter residue of mixed protein from synovial fluid, or of albumin, globulin, haemoglobin or lactoglobulin, good clots were formed on acidification. The dry weight and nitrogen content of these clots were the same as those of clots formed from synovial fluid containing a corresponding amount of hyaluronic acid. This showed that the hyaluronic acid had not lost its ability to form a normal mucin clot.

(4) *Ethanol-precipitated hyaluronic acid*

Preparation. The method of Ropes *et al.* (1947) was followed exactly. Initially the hyaluronic acid was precipitated three times; a part of the product was then precipitated twice more. The product was quite colourless; it dissolved easily to form a somewhat cloudy, viscous solution which became clearer on adding alkali.

Composition. The analytical results for the two samples are given in Table 3. The thrice-precipitated material closely resembled the undegraded ultrafilter residues in its composition, except that its glucosamine content was higher, as was that of the five-times precipitated sample; it gave a single boundary in the ultracentrifuge (Fig. 7*f*). Both samples contained protein, as is shown by the absorption at 285 mμ. and by the high values of total nitrogen; the five-times precipitated material contained less protein than the other and had a correspondingly low specific refractive increment.

Physical properties of synovial fluid and derived preparations

Viscosity, measured in the Ostwald viscometer

It was realized that the measured viscosity of material of this sort varies rapidly with the rate of shear and that the rate of shear in a capillary viscometer is non-uniform and varies with the flow time.

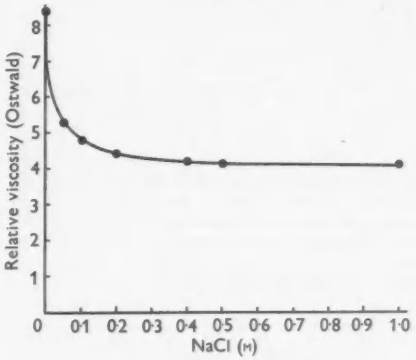

Fig. 3. Variation of relative viscosity (Ostwald) of synovial fluid with concentration of sodium chloride.

Nevertheless, reproducible results are obtainable when the conditions (viscometer, temperature, concentration of solute and composition of solvent) are constant, and are adequate for the comparison of different materials. The variation of the viscosity of a given solution with the concentration of salt (Fig. 3) agreed with the results of Ropes *et al.* (1947). For all subsequent measurements the standard buffer was used.

Synovial fluid. The logarithm of the relative viscosity varied linearly with concentration, values for different samples of fluid falling on the same line. This is shown in Fig. 4, in which relative viscosity values for mucin, ultrafilter residues and the ethanol-precipitated hyaluronic acid are also given for comparison.

Mucin. The viscosities of solutions of mucin tended to be lower than those of synovial fluid at corresponding concentrations of hyaluronic acid glucosamine, though in one case a value was obtained which agreed well. Repeated precipitation appeared to lower the viscosity further. The viscosity seemed to be a more sensitive index of degradation than the rate of sedimentation.

Ultrafilter residue. The viscosity of two samples of ultrafilter residue fell close to the values obtained for synovial fluid at similar concentrations. Degradation in samples 19*K* (*c*) and 19*K* (2) was accompanied by a marked loss in viscosity.

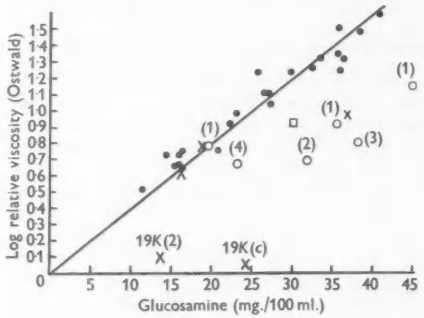

Fig. 4. Variation of the log of the relative viscosity (Ostwald) with concentration of hyaluronic acid glucosamine: ●, synovial fluid; ○, mucins (number of precipitations are shown in brackets); ×, ultrafilter residues (19*K* (2) and 19*K* (*c*) are marked); □, ethanol-precipitated hyaluronic acid (three precipitations). All preparations dialysed against standard buffer.

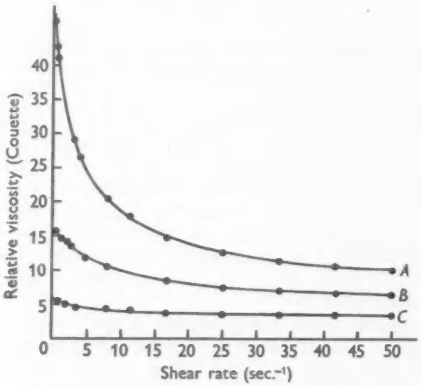

Fig. 5. Variation of relative viscosity (Couette) with rate of shear for different concentrations of synovial fluid, dialysed against buffer. Curve *A*, 21 mg. of hyaluronic acid glucosamine/100 ml.; curve *B*, 14 mg./100 ml., curve *C*, 7 mg./100 ml.

Ethanol-precipitated hyaluronic acid. The viscosity of the thrice-precipitated material was normal while that of the five-times precipitated material was somewhat low compared with synovial fluid and the ultrafilter residue at similar concentrations.

Viscosity, measured in the Couette viscometer

Synovial fluid. The relation of viscosity to rate of shear was studied in the Couette viscometer at different concentrations of synovial fluid. The curves (Fig. 5) of $\eta_{rel.}$ against rate of shear (r) were found to be approximately hyperbolic and to be expressed by

$$(r+a)\,(\eta_{rel.}-\eta_{rel.\,(\infty)})=\text{constant},$$

where a is a constant and $\eta_{rel.\,(\infty)}$ is the relative viscosity at infinite rate of shear. This relationship was used to obtain the values of $\eta_{rel.\,(\infty)}$, which were found to agree approximately with the values obtained in the Ostwald viscometer; the values of $\eta_{rel.}$

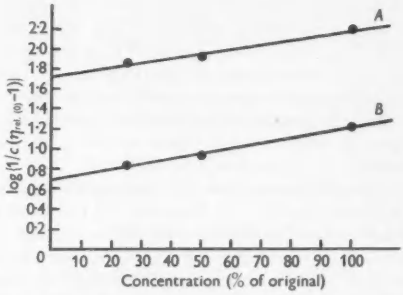

Fig. 6. Variation of the log $[1/c\,(\eta_{rel.\,(0)}-1)]$ (Couette) of synovial fluid with concentration. Curve *A*, dialysed against water; curve *B*, dialysed against buffer.

at zero rate of shear, $\eta_{rel.\,(0)}$, were obtained from the intercepts at $r=0$ of plots of $1/\eta_{rel.}$ against r. The values thus obtained of $\eta_{rel.\,(0)}$ were plotted against concentration as log $[1/c(\eta_{rel.\,(0)}-1)]$, and extrapolated to zero concentration (Fig. 6). Although the intrinsic viscosity, determined in this way, includes the contribution by serum protein, this is negligible.

Rate of sedimentation in the ultracentrifuge

Synovial fluid. The rate of sedimentation of the hyaluronic acid boundary, as a function of concentration, was studied in some detail. The results were plotted as $1/S_{20}$ (corr.) against concentration of hyaluronic acid glucosamine, and were found to be fairly reproducible from one sample of fluid to another. In Fig. 8 are shown values for the same sample of fluid as was used in the Couette viscometer, at various concentrations. This plot was approximately linear in the more dilute range, but was curved at higher concentrations. Extrapolation to zero concentration could be made with fair accuracy, the limiting value of S_{20} (corr.) being 13×10^{-13}. Fig. 8 also shows values of the sedimentation constant for different samples of mucin, ultrafilter residue and ethanol-precipitated hyaluronic acid.

At higher concentrations, the hyaluronic acid boundary in synovial fluid was extremely sharp (Fig. 7*a*) and remained so throughout the sedimentation run. At lower concentrations, however, it became more diffuse with time.

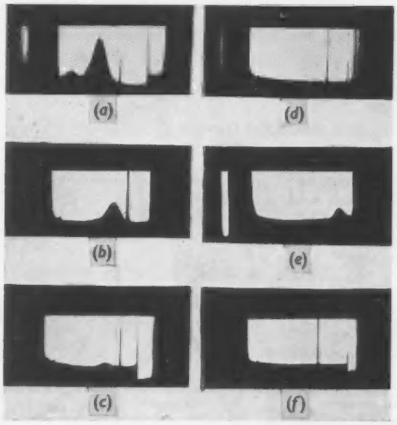

Fig. 7. Ultracentrifuge diagrams: (*a*) synovial fluid; (*b*) mucin; (*c*) ultrafilter residue with free globulin; (*d*) ultrafilter residue with no free protein; (*e*) ultrafilter residue, diffuse boundary due to degradation; (*f*) ethanol-precipitated hyaluronic acid.

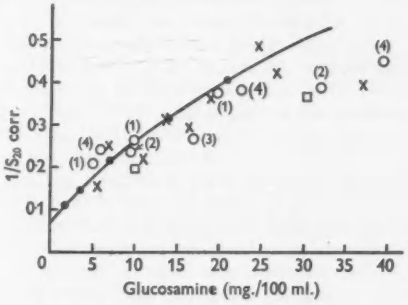

Fig. 8. Variation of sedimentation constant with concentration of hyaluronic acid glucosamine; ●, synovial fluid; ○, mucins (number of precipitations are shown in brackets); ×, ultrafilter residues; □, ethanol-precipitated hyaluronic acid (three precipitations).

Mucin. The sedimentation constant of the hyaluronic acid component of once-precipitated mucin appeared to be normal. Repeated precipitation, however, led to high values for the sedimentation constant (Fig. 8). Also, the sharpness of the boundary was impaired. The time taken for the boundary to become noticeably diffuse, as a function of concentration, could in fact be used as an accessory criterion of the state of the hyaluronic acid. This is shown in Fig. 9, in which results for synovial fluid, ultrafilter residue and ethanol-precipitated hyaluronic acid are also given. It is evident that at a given concentration a much longer time is taken for the boundary to become diffuse in synovial fluid or in once-precipitated mucin than in mucin precipitated several times.

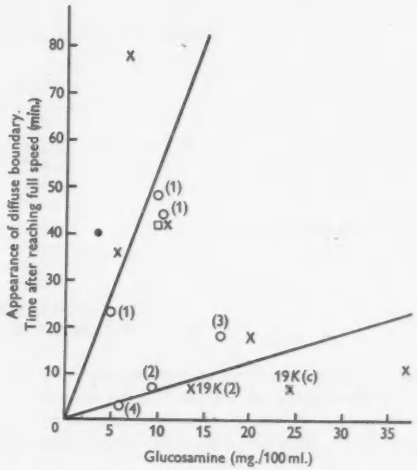

Fig. 9. Relation between concentration of hyaluronic acid glucosamine and time to appearance of diffuse boundary in ultracentrifuge. (The lower line represents materials in which degradation had taken place.) ●, synovial fluid; ○, mucins (number of precipitations are shown in brackets); ×, ultrafilter residues (19*K*(2) and 19*K*(*c*) are marked); □, ethanol-precipitated hyaluronic acid (three precipitations).

Ultrafilter residue. All samples of ultrafilter residue containing 7% of nitrogen or less showed only the typical hyaluronic acid component (Fig. 7*d*), which sedimented at the normal rate (Fig. 8). The sedimentation constants of those containing low proportions of nitrogen (19*K*(*c*) and 19*K*(2)) showed little departure from normal, but the boundaries of these samples showed a marked decrease in sharpness compared with normal (Figs. 7*e*, 9). The same abnormality was shown by an ultrafilter residue which had been prepared from a thrice-precipitated mucin.

Ethanol-precipitated hyaluronic acid. Only the thrice-precipitated material was used. The single sedimentation boundary was sharp, and showed only the presence of the hyaluronic acid component (Fig. 7*f*). Its sedimentation constant was a little higher than normal.

Diffusion

Mucin. Measurements were made on mucin 21 over a range of concentration and are given in Table 4 in terms of total refractive increment. Since mucin contains only two components, the hyaluronic acid complex and albumin, the diffusion record can be analysed for the diffusion constants and proportions of these components by the method of Ogston (1949). Even with the special cell, described above, difficulty was too low for the diffusion record to be analysed in terms of two components. However, the proportion of protein, in terms of refractive increment, was known to be 40 %, from the sedimentation data; the diffusion constant of this protein was found, in a separate experiment, to be $4{\cdot}0 \times 10^{-7}$, consistent with its being globulin. Accordingly, the diffusion constant of the hyaluronic acid complex could be obtained from the measured diffusion constant. The results are given in Table 4 and Fig. 10.

Table 4. *Diffusion constants of mucin and ultrafilter residue*

Material	Refractive fraction of hyaluronic acid complex	Refractive increment of hyaluronic acid complex $\times 10^5$	$D_{20} \times 10^7$ Hyaluronic acid complex	$D_{20} \times 10^7$ Protein
Mucin 21	0·40*	52	1·17	4·9
	0·43*	36	0·87	5·2
	0·40*	27	1·12	5·9
	0·33*	22	0·97	4·4
Ultrafilter residue 22*K*	0·605†	11·6	0·92	—
	0·605†	8·7	1·07	—
	0·605†	6·7	1·34	—
	0·605†	3·9	1·67	—

* Estimated from the diffusion record.
† Estimated from the ultracentrifuge diagram.

was experienced in forming satisfactory boundaries, so that the results are not likely to be very accurate. Nevertheless, they are reasonably consistent (Table 4 and Fig. 10); the proportion of components agrees

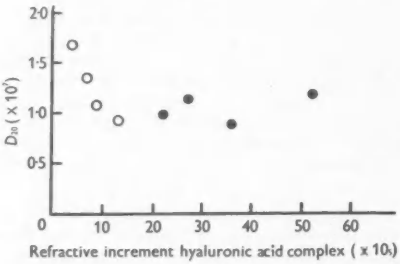

Fig. 10. Diffusion constant of hyaluronic acid complex for different concentrations of mucin and ultrafilter residue: ●, mucin; ○, ultrafilter residue.

with that estimated from the sedimentation diagram of this material (Table 2 and Fig. 7*b*) and the diffusion constant of the protein component is of the order of magnitude expected for albumin (D_{20} in this buffer should be about $5{\cdot}7 \times 10^{-7}$; Kekwick, 1938). The diffusion constant of the hyaluronic acid complex is evidently of the order of 1×10^{-7} and does not vary considerably with concentration.

Ultrafilter residue. Measurements were made on sample 22*K*. The highest concentration at which this material would form a boundary in the diffusion cell

Ultraviolet absorption

Ultrafilter residue and ethanol-precipitated hyaluronic acid. The absorption curves of several samples of ultrafilter residue and ethanol-precipitated hyaluronic acid were determined between 240 and 400 mμ. (Fig. 11). All show a hump or maximum in the region of 275 mμ. in neutral or acid solution, with a shift in the position of the maximum to about 285 mμ. in 0·1N-NaOH. This type of absorption curve is typical of proteins and is due, mainly, to the tyrosine and tryptophan which they contain (Holiday, 1936; Sizer & Peacock, 1947). An accurate analysis of the absorption curves in terms of tyrosine and tryptophan could not be made because of their low extinction compared with the non-specific scattering due, presumably, to the polysaccharide; the solutions were never quite clear even in alkali. Analysis by the method of Holiday & Ogston (1938) gave values for tyrosine of 1·3–1·7 % and for tryptophan of 0·4–1·0 % of total protein.

DISCUSSION

Methods of separating undegraded hyaluronic acid from synovial fluid

Definite relations were found to hold in synovial fluid between the concentration of hyaluronic acid glucosamine and the relative viscosity, rate of sedimentation and sharpness of the sedimentation boundary. The occurrence of similar relations in its derivatives was taken as evidence that they had been

prepared in an undegraded state. It was found possible to obtain undegraded preparations containing hyaluronic acid by ultrafiltration, by precipitation as mucin and by precipitation with ethanol.

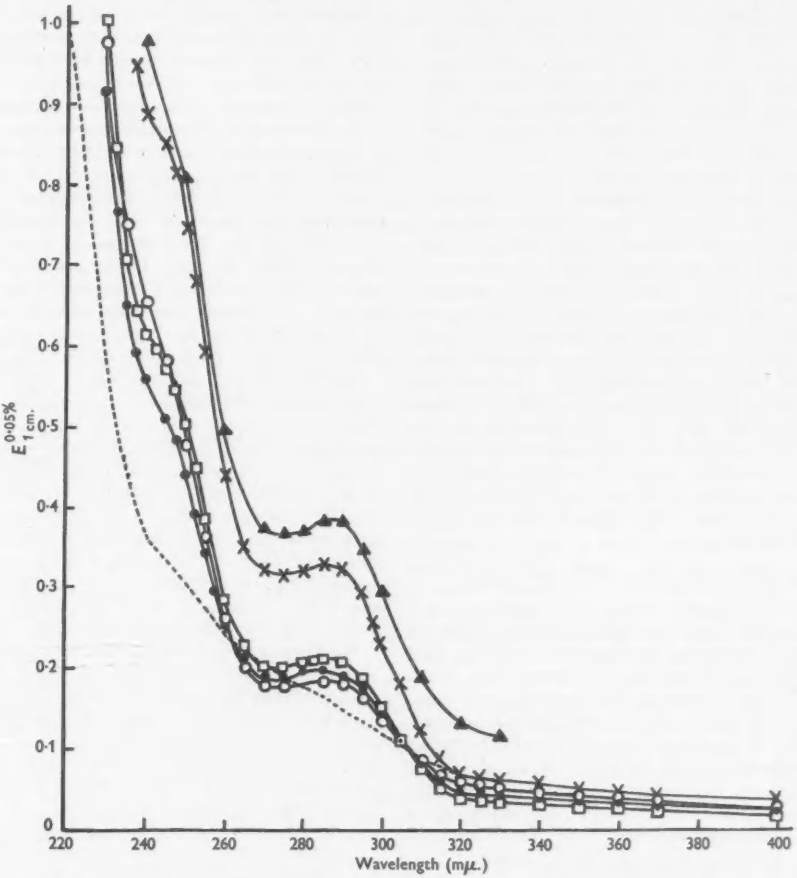

Fig. 11. Ultraviolet absorption of ultrafilter residues and of ethanol-precipitated hyaluronic acid in 0·1 N-NaOH: ×, ultrafilter residue 19*K*(1); □, ultrafilter residue 19*K*(2); ●, ultrafilter residue 19*K*(*c*); ○, ultrafilter residue 16*K*; ▲ three-times precipitated hyaluronic acid; - - -, five-times precipitated hyaluronic acid.

A single precipitation of mucin often caused degradation and repeated precipitation always did so; repeated precipitation of mucin with ethanol could cause degradation; in some cases, the product obtained by ultrafiltration had become degraded. Evidently precipitation of mucin is not a satisfactory first stage in the separation.

All the undegraded preparations which we made, except ultrafilter residue 16*K*, contained 7 % of total nitrogen or more; any excess of the total nitrogen over 7 % was always accompanied by the presence of free protein, which sedimented as a separate component. Those preparations which contained 7 % of total nitrogen or less showed the presence of only a single component in the ultracentrifuge. All preparations which contained less than 7 % of total nitrogen, except ultrafilter residue 16*K*, showed signs of degradation. We therefore conclude that the combination of hyaluronic acid with other nitrogenous material is essential to the maintenance of the physico-chemical state in which it occurs in synovial fluid.

The methods which have been used by previous workers for the separation of hyaluronic acid have

been, for the most part, relatively vigorous. Most of them have involved an initial precipitation of mucin, followed by removal of protein by shaking with chloroform and amyl alcohol (Blix & Snellman, 1945; Meyer *et al.* 1939), by use of zinc acetate (Meyer *et al.* 1939), or by digestion with trypsin (Ropes *et al.* 1947). Final isolation of the hyaluronic acid has usually been by precipitation with ethanol. Ropes *et al.* (1947) eliminated protein and precipitated hyaluronic acid in one operation by precipitating from an alkaline solution of mucin with ethanol at $-20°$. Hadidian & Pirie (1948) shook with pyridine in the presence of ammonium sulphate.

Many workers have measured the viscosities of hyaluronic acid solutions, but at various concentrations and in various buffers (reviewed by Hadidian & Pirie, 1948). This fact, together with the variety of the sources from which the hyaluronic acid was prepared, makes comparison difficult. However, our best samples of ultrafilter residue have a viscosity equal to that of synovial fluid at corresponding concentration. This corresponds to a relative viscosity of 39 at a concentration of 1 g. of hyaluronic acid per litre (0·4 mg. of hyaluronic acid glucosamine per ml.) in 0·2M-NaCl, and this value is considerably greater than any published value for isolated hyaluronic acid. This suggests that by the methods previously used hyaluronic acid was isolated only with some degree of degradation.

Composition of the hyaluronic acid complex

(*a*) *Protein.* It has been pointed out above that the undegraded hyaluronic acid complex, separated from synovial fluid by ultrafiltration, contains 7 % of total nitrogen. Of the total nitrogen, 5 % is unaccounted for by glucosamine present in the complex; the presence of amino-acids in the complex, shown by its ultraviolet absorption and by paper chromatography, suggests that this excess of nitrogen is contributed by protein. The specific refractive increments of protein, hyaluronic acid and complex are also consistent with this view. Analysis of the sedimentation diagram of mucin and of its diffusion record have also shown that part of the protein present is bound to hyaluronic acid, to form a complex of the same composition as ultrafilter residue (Table 5). Other workers quoted above have shown that hyaluronic acid can be obtained free from protein, but only by use of relatively severe methods. Blix (1940) and Hesselvik (1940) did obtain protein-free hyaluronic acid by electrophoresis, but they applied no physico-chemical tests to their products.

(*b*) *Glucosamine.* Equimolar proportions of acetylglucosamine and glucuronic acid in hyaluronic acid require a glucosamine percentage of 42·6. However, a wide range of values has been reported, of which most have been lower than this. For example, Meyer *et al.* (1939) give values between 34·5 and 43·7 for synovial hyaluronic acid. Ropes *et al.* (1947) give a value of 40 %. Hadidian & Pirie (1948) give values of 34–38 % for viscous preparations from umbilical cord and 20–25 % for non-viscous preparations. Blix & Snellman (1945) give a mean value of 33 %.

Our analysis of hyaluronic acid, prepared by repeated ethanol precipitation, gave values near the theoretical. On the other hand, the proportion of glucosamine found in the non-protein fraction of the ultrafilter residue was low (Table 3). The estimation of glucosamine has not been entirely satisfactory, owing to the possibility of its destruction during hydrolysis; but a mean allowance has been made for this destruction in calculating the proportion of glucosamine and allowance for the maximum destruction found would still not, in most cases, give the theoretical proportion of glucosamine.

If a value of 25 % for the proportion of glucosamine in the whole complex is accepted (Table 3), and if it is assumed that glucosamine constitutes 42·6 % of hyaluronic acid, two alternative assumptions may be made about the composition of the non-hyaluronic acid part of the complex, which contributes 5 g. of nitrogen/100 g. of complex (Table 6). These are that (*a*) the protein contains 15·5 % of nitrogen, in which case some third component free from nitrogen and

Table 5. *Proportion of hyaluronic acid complex in mucin, ultrafilter residue and ethanol precipitate, and its content of hyaluronic acid*

	Mucin			Ultrafilter residue			Ethanol-precipitate (3 precipitations)
	12*K*	18*A*	21	19*K*(1)	22*K*	23	23
	Proportion of complex in total material						
Refractive fraction*	0·34	0·35	0·36	Approx. 1·00	0·605	1·00	1·00
Weight fraction†	0·38	0·39	0·40	Approx. 1·00	0·64	1·00	1·00
	Proportion of hyaluronic acid in complex						
Refractive fraction*	0·71	0·73	0·65	0·63	0·64	0·58	0·66
Weight fraction†	0·76	0·775	0·705	0·68	0·68	0·63	0·71

* I.e. fraction contributed to the total refractive increment.
† Calculated from the refractive fraction by use of the appropriate specific refractive increments.

glucosamine is also present, or (*b*) the protein contains only about 12% of nitrogen and no third component is present. On the other hand, a higher glucosamine content (29%) would make it unnecessary to assume the presence of either a third component or protein of an abnormally low nitrogen content. In any case, the presence of protein constituting at least 30% of the complex is required.

Table 6. *Possible compositions of hyaluronic acid complex, containing 25% glucosamine and 5% non-glucosamine nitrogen*

Components as % of total	Assuming protein contains 15·5% N	Assuming protein contains 12·2% N
Hyaluronic acid	59	59
Protein	32	41
Third component	9	0

Physico-chemical properties

All our results suggest that the particles of the hyaluronic acid complex are very large and asymmetric. The filtration experiments indicate a particle of length between 0·2 and 0·5 μ.

Sedimentation and diffusion. The value of S_{20} (corr.) at zero concentration was found to be 13×10^{-13}. The diffusion results obtained with mucin and with ultrafilter residue (Table 4 and Fig. 10) agree moderately well, considering the technical difficulty of these measurements. Extrapolation to zero concentration is made uncertain by the apparent steep rise of the diffusion constant at the lowest concentrations. We have ascribed this rise to experimental error and have taken the limiting value of D_{20} (corr.) to be $1{\cdot}0 \times 10^{-7}$, on the grounds (Singer, 1947) that the diffusion constants of asymmetric particles vary little with concentration in other cases and that this is in accordance with theoretical expectation.

Using thesel imiting values of S_{20} (corr.) and of D_{20} (corr.) and assuming a value of 0·69 for the partial specific volume, $\bar{V}$ (taking the complex to consist of 0·35 of protein and 0·65 of hyaluronic acid, having partial specific volumes 0·75 and 0·65 respectively) we obtain (Svedberg & Pedersen, 1940):

$$M = \frac{RTS}{D(1-\bar{V}\rho)} = 1{\cdot}0 \times 10^{6},$$

and

$$f/f_0 = 10^{-8}\left(\frac{1-\bar{V}\rho}{D^2 S\bar{V}}\right)^{\frac{1}{3}} = 3{\cdot}25.$$

From the relationship of Perrin (1936), this value of f/f_0 would be satisfied if the particles were elongated ellipsoids (unsolvated) having an axial ratio of 62. From this axial ratio, the particle weight and the partial specific volume, the dimensions of the particles would be: length 0·2 μ.; thickness 3·3 mμ., which agree with those indicated by ultrafiltration.

Viscosity. The value of the intrinsic viscosity at zero rate of shear, derived from Fig. 6 and expressed as

$$V_0 \equiv \frac{1}{\phi}\left(\frac{\eta}{\eta_0} - 1\right) \quad \phi \to 0,$$

where ϕ is the volume fraction of the hyaluronic acid complex, is $1{\cdot}36 \times 10^4$. This quantity can be used to determine the asymmetry of the particles by means of the relationship of Simha (1940), computed by Mehl, Oncley & Simha (1940). The axial ratio obtained, assuming that the particles are elongated and unsolvated, is 590.

This discrepancy between the indications of sedimentation and diffusion and of viscosity cannot be resolved by any assumption about the solvation of the particles and is more serious if the particles are assumed to be disk-shaped rather than rod-shaped. As a tentative explanation, we may suppose that the particles are interconnected to form a loose, sponge-like structure; the processes of sedimentation and diffusion involve only compression and expansion of this structure, which would not necessarily cause any widespread breaking of interparticle links; on the other hand, these links might contribute largely to the shearing force required to cause viscous flow and would thus increase the viscosity and the apparent asymmetry of the particles derived from it.

It is seen from Fig. 6 that the relative lowering of the viscosity by salt is nearly independent of the concentration of hyaluronic acid. The effect of salt should be to reduce the thickness of the electrical double layer surrounding the particles and so to reduce their interaction both with solvent and with each other. The effect on viscosity of interaction with solvent should not vary much with the concentration of hyaluronic acid, whereas the interparticle interaction should vary rapidly with concentration. The results suggest, therefore, that salt affects the former rather than the latter, and hence that the interparticle interaction is not mainly ionic in character.

The fact that treatment of the hyaluronic acid complex affected the viscosity more markedly than it affected the rate of sedimentation suggests that the first degradative changes involve a reduction of interparticle linking.

CONCLUSION

Evidence has been produced to show that hyaluronic acid occurs in synovial fluid in the form of a definite complex with protein and, perhaps, with another material which contains neither glucosamine nor nitrogen. Isolation of this complex by ultrafiltration gives an undegraded product: it appears, however, that other methods of separation are liable to cause degradation and, in particular, any removal of protein from the complex causes significant changes in its physico-chemical properties. The particle weight

of the complex is about 10^6; the particles are probably rod-shaped with a length of 200 mμ. and a thickness of 3·3 mμ.

SUMMARY

1. A hyaluronic acid complex was prepared from synovial fluid by ultrafiltration, and judged to be undegraded by viscosity and ultracentrifuge measurements.

2. Its composition and physico-chemical properties were compared with those of the native synovial fluid, mucin, and hyaluronic acid prepared from mucin by ethanol precipitation at $-20°$.

3. Hyaluronic acid occurs in synovial fluid in close association with protein, which constitutes about 30 % of the complex; removal of this protein leads to degradation.

4. The particle weight of this complex, estimated by sedimentation and diffusion, is about 10^6, with an axial ratio of about 60.

5. The viscosity at low rates of shear indicates considerable interaction between these 'particles', even at high dilution.

6. The hyaluronic acid was in most cases found to contain somewhat less than the theoretical percentage of glucosamine.

We are grateful to Messrs R. R. Alden and Son, Oxford, for facilities at their Eastwick Farm slaughterhouse; to Mr J. T. Cox for his help in the design of, and his construction of, the Couette viscometer; to Mr D. S. Parsons for making a paper chromatogram. One of us (J. E. S.) is indebted to the Medical Research Council for a Training Studentship.

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